ROLES OF TEA CONSUMPTION & CO-FORTIFICATION IN IRON NUTRITION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2012

ROLES OF TEA CONSUMPTION & CO-FORTIFICATION IN IRON NUTRITION

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Cornell University 2012

Objective I: Develop extruded rice capable of delivering iron, zinc, vitamin A and ascorbic acid, without developing unwanted sensory changes. Investigate if vitamin A increases iron dialyzability.

Results: Multinutrient fortification with ferrous sulfate or micronized ferric pyrophosphate (mFPP) resulted in a totally unacceptable product with noticeable darkening of the extruded kernels. Fortification with mFPP alone, produced kernels similar to those produced with electrolytic iron (EF) which had very little darkening regardless of whether additional minerals were added. *In vitro* digestion of the mFPP and EF samples revealed an increase in iron dialyzability with the addition of ascorbic acid or ascorbic acid and vitamin A together. The addition of Vitamin A did not increase iron dialyzability from corn porridges.

Objective II: Examine the effect of green or black tea on iron absorption in animal models.

Methods:

Rats: Thirty-six weanling rats were allocated into 3 groups. Control group was fed a semi-purified diet (20mg Fe/kg diet), Oral group was fed green tea extract mixed into the diet (28.6g tea/kg diet), and Gavage group was fed control diet with a twice daily gavage of

tea solution (0.25g tea/ml). Saliva was collected on day 8 or day 31. Iron absorption was assessed using a $^{58}Fe^{3+}$ tracer administered on day 1 or day 24.

Pigs: Weanling piglets were allocated into 3 groups. Control group was fed an iron-deficient corn-soybean diet (46mg Fe/kg diet). The tea groups were fed the control diet with black or green tea extract mixed into the diet (10g tea/kg diet). Saliva was collected weekly. Iron absorption was assessed by hemoglobin repletion efficiency (HRE). Parotid glands were analyzed for proline-rich proteins (PRP) mRNA concentration.

Results:

Rats: There was no significant difference in iron absorption between the three groups on either day 1 or 24. Salivary PRPs increased to a greater extent in the oral group than in the gavage group, compared to the control.

Pigs: There was no significant difference in iron absorption between the three groups. PRP mRNA concentration was not different among the three groups.

Conclusions: Tea does not decrease iron absorption in rats or piglets, but does affect the saliva proteome. Our results indicate that either tea does not significantly inhibit iron absorption or that animals are able to quickly adapt to any inhibitory effects of tea.

BIOGRAPHICAL SKETCH

Ariel Bianca Beverly was born in 1984 to Dr. Roland and Jacquelynn Beverly. She has one sister, Corinne. She grew up in Southern California, attending St. Margaret's Episcopal School from 3rd grade until graduating from the Upper School in 2002. Ariel pursued her undergraduate education at Saint Mary's College of California in Moraga, California. At Saint Mary's, Ariel served for 3 years on the Academic Honor Council and was active in campus programming as the Program Board Vice-President. She performed senior research on porphyrins under the guidance of Dr. Steve Bachofer. In 2006, Ariel graduated cum laude from Saint Mary's with a Bachelors of Science in Chemistry.

Ariel entered the Ph.D. program in Food Science at Cornell University in 2007. There, she pursued an interest in food chemistry and iron nutrition under the guidance of Dr. Dennis Miller. While at Cornell, Ariel was active in planning the Expanding Your Horizons Conference as the Food Chair for 2 years.

To Mom, Dad & Corinne,
with love and gratitude

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Dennis Miller, for his guidance and support as I progressed through my Ph.D. program. Even despite your busy schedule, you always made time to meet with me and assist in any way that you could. At times when I felt a little frazzled, your calm presence and wise words of advice provided the encouragement and motivation I needed to stay focused. I am also grateful to Dr. J. Thomas Brenna and Dr. Xingen Lei for their time as members of my committee and for their careful review and critique of my project proposal and my thesis. I send heartfelt thanks to my unofficial committee member, Dr. Alicia Orta-Ramirez—thank you for always being there to support me, to chat with me and to encourage me. I would not have gotten this far without you.

Many people contributed to the research in this thesis. First, Dr. Leanne Zhu planned and performed the rat study and when she left, she graciously allowed me to complete the analysis and write the manuscript. Dr. Ted Thannhauser and his staff, Kevin Howe and Tara Fish, were instrumental in the analysis of the pig and rat saliva. A special note of gratitude is extended to Tara, who always demonstrated patience and diligence in working with our saliva samples and also in teaching me how to complete the various proteomics techniques. Dr. Elad Tako shared his lab and expertise with us during the analysis of the parotid gland mRNA. Mary S Bodis and Pei Pei Chang performed the mineral analysis. Karl Roneker and the staff of the Cornell Swine Research Farm were indispensable during the pig studies, as they cared for the animals and assisted us in sample collection. Dr. Xingen Lei's lab warmly accepted me and shared their lab space and expertise as I completed the microbial population analysis, especially Lvhui Sun, who directly mentored me in the procedures of TRFLP analysis. Stephanie Ko performed the serum iron measurements as her research project. Hemalatha Ganapathyswamy, Thangavel Kulandaiswamy and Sean Schell prepared the extruded rice samples. I am also grateful to my labmate, Claudia Pazlopez, who was always a comforting presence in lab, and to Janette

Robbins, who helped me survive my time at Cornell by always saying the right thing at the right time. Last, but certainly not least, I reserve the sincerest gratitude for my labmate Dr. Tong Li, who not only spent hours catching and restraining pigs with me, but gave up her time to assist with sample analysis whenever I needed help. Tong, I will always remember the conversations, the laughs and most certainly, the dumplings.

Deepest love and thanks are extended to all of my family and friends. There are too many of you to list, but know that I am ever so grateful. Your love and support are what made this possible. I am ever so thankful to Akina and Laura for being awesome roommates that let me write my thesis in peace. To Meg, for offering to read and edit my thesis…and for the vampires. To Chester, for your constant companionship. To my sister, Corinne, for always eating my cooking; it is you that taught me to be confident in my abilities. Lastly, to my parents who never ceased to remind me, "Be strong and very courageous" and "You're a winner!"; you are the best role models that anyone could ask for. I love you all.

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CHAPTER 1: LITERATURE REVIEW

1.1 Iron Nutrition

Iron is an important and essential element for biological systems. Within humans, and other animals, the primary use of iron is for oxygen transport as a part of the protein complex, hemoglobin. In fact, for humans approximately two-thirds of the body's iron is contained in hemoglobin. The remaining third is either found in myoglobin and other heme proteins (e.g., *cyctochromes*, *catalase)*, bound to transferrin in the blood, or stored within ferritin for later use. An average adult male (70 kg) has a body iron content of approximately 4g, with 1800 mg of iron in red blood cells, 600 mg of iron stored in reticuloendothelial macrophages and another 300 mg in bone marrow for red blood cell synthesis (Hentze *et al.* 2004). Additionally, about 4 mg of iron is bound to transferrin in the blood, 1000 mg is stored in the liver and 400 mg is utilized by other cells and tissues (Hentze *et al.* 2004). On average, a man will lose approximately 1 mg of iron per day, mainly from sloughed skin and intestinal cells but small amounts are also lost in sweat, urine and bile (Bothwell *et al.* 1979). A woman in her child bearing years will lose an additional 0.5 to 1 mg of iron per day due to menses (Conrad & Umbreit 2000). Also, women tend to have lower liver iron stores, averaging only 300 mg compared to the reported value of 1000 mg in men (Knutson 2010). On average, the body will absorb only 1-2 mg of iron from food each day (Hentze *et al.* 2004). The Recommended Dietary Allowances (RDA) for iron (Institute of Medicine 2006) assume that only 18% of consumed iron will be absorbed by non-pregnant women, men, adolescents and children over age 1, 10 % of consumed iron by infants under age 1 and 25% by pregnant women. The RDA are 11 mg for adolescent males (14-18 years), 8 mg/day for

adult males (19+ years) and post-menopausal women, 15 mg/day for adolescent females (14-18 years), and 18 mg/day for pre-menopausal women. Additionally, pregnant women should ingest 27 mg/day of iron, but lactating women only 10 mg/day.

1.1.1 Mechanisms of Iron Absorption

While iron is critical for many metabolic processes, it is also quite toxic to the body because it readily catalyzes the formation of free radicals, causing tissue damage (McCord 1998). As such, iron is always found bound to proteins to protect against the formation of these free radicals (Conrad & Umbreit 2000). Additionally, the body does not have a mechanism to eliminate excess iron, and therefore to maintain homeostasis, iron absorption is tightly controlled. Dietary iron is present in two forms: heme iron, which is found in meat, or nonheme iron, which comes from foods of plant origin. Absorption of dietary nonheme iron from a meal ranges from 1% to 20% (Bothwell *et al.* 1979), while the absorption of heme iron is reported to be higher, around 15-35% (Carpenter & Mahoney 1992). Heme iron only contributes 10-15% of the total iron intake in developed countries, but because it is more readily absorbed, it is estimated to contribute ≥40% of total absorbed iron (Carpenter & Mahoney 1992; Hunt 2002).

Most dietary nonheme iron is present either in the ferric (3+) or the ferrous (2+) form, the only two iron oxidation states that are stable in aqueous solutions (Spiro & Saltman 1974). Within acidic environments (pH ≤ 1), both ferrous and ferric iron are soluble, forming the hydrated complexes $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$, respectively. At slightly higher pHs (pH ≥ 2), ferric iron becomes less soluble, tending to irreversibly form

hydroxide ion complexes, such as $Fe(OH)^{2+}$, $Fe(OH)_2^{+}$, and $Fe_2(OH)_2^{4+}$. In time, these complexes will polymerize, forming a ferric-hydroxyl gel (Schneider & Schwyn 1987). At physiologically important pHs, ferric iron is almost completely insoluble and it must be chelated by mucins and dietary ligands in the stomach in order to remain in solution within the duodenum, the primary site for iron absorption (Conrad *et al.* 1991). While most iron is absorbed within the duodenum and upper jejunum, it has been proposed that some iron may also be absorbed in the colon (Blachier *et al.* 2007).

In the duodenum, ferric iron can be reduced by the brush border ferric reductase duodenal cytochrome b (McKie *et al.* 2001) or by dietary components such as ascorbic acid (Lynch & Cook 1980). Since only ferrous iron can be taken up by enterocytes, ferric iron must be reduced prior to absorption. Once in the ferrous form, iron is moved into the duodenal absorptive cell (enterocyte) by divalent metal transporter 1 (DMT1), a trans membrane transporter protein. Presumably there are intracellular iron transporters as well, but only one has been identified. The protein poly (rC)-binding protein 1 (Pcbp1) can bind up to three atoms and deliver them to ferritin, a large iron storage protein (Shi *et al.* 2008). Suppression of this protein results in a decreased amount of iron stored within ferritin and increased cytosolic iron concentrations.

Intracellular iron is transferred out of the enterocyte by ferroportin, the only known export protein (Donovan *et al.* 2005; Ganz 2005). When transferred out of the cell, the iron is oxidized from the ferrous to the ferric state by the protein hephaestin and immediately the iron is bound to the protein transferrin for transport in the blood plasma. A similar export process occurs in hepatocytes and macrophages that contain storage iron; the main

difference is that ceruloplasm, instead of hepahestin, is the ferroxidase protein (Hellman & Gitlin 2002).

The process of how heme iron is absorbed into the enterocyte is not as well characterized as the process for nonheme iron, but it is believed that heme iron has a separate transporter. The two proposed hypotheses to explain the mechanism of iron uptake into the enterocyte are 1) heme is transported across the brush border membrane by a heme carrier protein 1 (HCP1) and 2) heme iron enters the enterocyte by receptor-mediated endocytosis (Shayeghi *et al.* 2005; West & Oates 2008). Outside the cell, enzymes degrade hemoglobin and myoglobin, releasing the heme complex. The heme travels into the enterocyte as an intact metalloporphyrin, but within the cell, the enzyme heme oxygenase, splits the porphyrin ring, releasing the iron (Conrad & Umbreit 2000). Once the iron is within the enterocyte, it enters the cellular iron pool and may be stored within ferritin or exported to the blood stream, the same as for nonheme iron.

As recently as 20 years ago, the mechanisms by which the body regulates iron absorption in response to the individual's iron status were unknown (Cook 1990). It was known that iron content in the body is tightly controlled to ensure appropriate levels of absorption to offset loses without toxic accumulation of excess iron. As well, it was understood that the body increases iron absorption during conditions of iron deficiency and decreases it in conditions of iron overload. However, within the last 10 years, scientists have begun to uncover the mechanisms of iron regulation. It is now known that the peptide hormone hepcidin maintains iron homeostasis by controlling the efflux of iron from enterocytes, macrophages and hepatocytes. Hepcidin binds to ferroportin, causing the

ferroportin to be internalized into the cell and degraded, thereby inhibiting the cells' ability to export iron (Nemeth *et al.* 2004). Hepcidin, which is synthesized in the liver and secreted into the circulation, is a small protein, only 25 amino acids in length (Krause *et al.* 2000; Park *et al.* 2001). Pigeon et al (2001) were the first to link hepcidin expression with iron status, noting that in iron overload hepcidin expression is high. Current research suggests that the body senses plasma iron based on two distinct but possibly connected pathways (Knutson 2010).

Briefly, the first pathway is based on interactions between bone morphogentic protein 6 (Bmp6) and its hepatocyte-surface receptor, Bmpr (bone morphogentic protein receptor). Under iron replete conditions, Bmp6 interacts with Bmpr and another surface protein, hemojuvelin (Hjv), causing an intracellular signaling cascade that activates hepcidin transcription (Papanikolaou *et al.* 2004; Lin *et al.* 2007; Truksa *et al.* 2009). Under iron replete conditions, levels of the protease furin increase and furin cleaves Hjv's glycosylphosphatidyinositol (GPI) tail resulting in an increase of extracellular soluble-Hjv (Silvestri *et al.* 2008a; Silvestri *et al.* 2008b). The soluble-Hjv (s-Hjv) sequesters available Bmp6, thereby preventing it from binding with Bmpr and Hjv to start the hepcidin transcription signaling cascade (Lin *et al.* 2005; Babitt *et al.* 2007).

The second pathway involves interactions between the human hemochromatosis protein (Hfe) and transferrin receptor proteins (Vujic Spasic *et al.* 2007; Vujic Spasic *et al.* 2008; Schmidt *et al.* 2008). During states of low transferrin saturation, Hfe associates only with transferrin receptor 1 (Tfr1) on the surface of hepatocytes (Schmidt *et al.* 2008). However, in situations of high Tf saturation, diferric Tf competes better than Hfe for similar

binding sites with Tfr1 and diferric Tf displaces Hfe (Bennett *et al.* 2000; Giannetti & Bjorkman 2004). The displaced Hfe moves to bind with the hepatocyte-specific transferrin receptor 2 (Tfr2,) producing a complex that signals the cell to synthesize and release hepcidin (Schmidt *et al.* 2008). It has been proposed that both pathways may join at a common point along the signaling cascade (Nemeth 2008; Wallace *et al.* 2009), but more research is needed to discern the exact signaling cascades for each pathway.

Under normal conditions, this process is how the body maintains iron homeostasis. Excess iron stimulates hepcidin synthesis which prevents iron from being released to the blood stream. However, inflammation can also stimulate hepcidin expression, causing anemia of chronic disease, even when iron stores may be adequate (Pigeon *et al.* 2001; Weinstein *et al.* 2002). At the other extreme, mutations of Hfe, Tfr2 and other sensing-related proteins, can cause these regulatory processes to function incorrectly, resulting in hereditary hemochromatosis, a disorder of excess iron accumulation. In persons with hereditary hemochromatosis, toxic levels of iron can cause iron-induced organ damage, such as diabetes, hypothyroidism, heart failure, destructive arthritis and liver disease (Montosi *et al.* 2001; Pietrangelo 2004; Pardo Silva *et al.* 2010). Nutritional deficiencies of vitamin A and riboflavin can also lead to decreased iron absorption (Fairweather-Tait *et al.* 1992; Hurrell & Hess 2004).

1.1.2. Nonheme Iron Bioavailability

Iron bioavailability is the amount of ingested iron that is available for use in body processes or for storage in the liver, bone marrow, spleen and other tissues. Iron

bioavailability has been described by Fairweather-Tait *et al.* (2005) as being dependent on a series of steps: 1) the release of iron from the food matrix in the gastrointestinal tract *(availability)*, 2) transport into the intestinal enterocytes *(uptake)*, 3) transport out of the enterocytes to the blood *(absorption)*, 4) transport to tissues for use *(utilization)* and 5) effective transport to storage sites *(body stores)*. Both food and host factors can influence this sequence of events.

As discussed in the previous section, the major factor affecting iron absorption is the iron status of the individual, barring any inflammation or disease. However, the presence of dietary enhancers and inhibitors can also contribute to an individual's ability to absorb nonheme iron (heme iron is much less dependent on dietary factors). The most common inhibitor in a plant-based diet is myo-inositol hexakisphosphate or phytate (Hallberg *et al.* 1989), but plant polyphenols can also decrease iron absorption from a meal (Hurrell *et al.* 1999). Iron binds to both phytate and polyphenols in the gut, preventing the iron from being taken up at the brush border membrane. A potent enhancer of iron absorption is ascorbic acid, which has been shown to increase absorption of iron from a meal, even partially overcoming the inhibitory effects of phytate (Hallberg *et al.* 1989) and polyphenols (Siegenberg *et al.* 1991). The other main enhancer of nonheme iron absorption is meat. The addition of meat to a plant-based meal increases the amount of nonheme iron absorbed (Bjorn-Rasmussen & Hallberg 1979; Baech *et al.* 2003). The mechanism for the enhancing effect of meat on absorption is unknown but possibly the digestion products of myofibrillar (muscle tissue) proteins reduce and chelate the iron, keeping it in solution in the duodenum and making it available for absorption (Storcksdieck Bonsmann & Hurrell 2007). It has also

been suggested that the globin degradation products (from the removal of heme from the hemoglobin complex in the duodenum), can also increase the bioavailability of nonheme iron (Conrad & Umbreit 2000).

Persons consuming large amounts of iron absorption inhibitors and low amounts of ascorbic acid coupled with low dietary iron intake are at highest risk for iron deficiency. Iron deficiency is a condition where the body has no mobilizable iron stores, resulting in insufficient iron to support normal functions. Prolonged iron deficiency can lead to iron deficiency anemia, which is a condition where the blood concentration of hemoglobin is below a healthy level and is usually accompanied by physiological signs of extreme deficiency. Iron deficiency can cause increased weakness and fatigue, resulting in the inability to concentrate and decreased work productivity (Scrimshaw 1990; Ballin *et al.* 1992). Children and infants with iron deficiency show delayed physical and behavioral development and impaired cognitive performance (Walter *et al.* 1983; Soemantri *et al.* 1985; Lozoff 1989; Lozoff *et al.* 1991; Pollitt 1997). Iron deficiency in pregnancy increases the risk of perinatal mother and infant death (Scholl & Hediger 1994; WHO 2008) and often results in a newborn with insufficient iron stores (Zimmermann & Hurrell 2007). Iron deficiency also impairs the immune system (Srikantia *et al.* 1976), possibly explaining why populations with high occurrence of iron deficiency have increased rates of morbidity from infectious disease (Fortuine 1966; Brown *et al.* 1967; Basta *et al.* 1979; Scrimshaw 1990).

Iron deficiency is a major problem worldwide because it affects not only developing countries but developed ones as well. The populations most at risk for iron deficiency and iron deficiency anemia are menstruating women, due to menses; children and adolescents,

due to rapid growth; and pregnant woman, due to increased maternal and placental blood volume and iron accumulation in the developing fetus. The World Health Organization (WHO) estimates that 40-50% of children under 5 and pregnant women in developing countries are iron deficient and that around 10% of those in developed countries are also iron deficient (WHO 2008).

The goal of this project was to explore two sides of addressing the global issue of iron deficiency. The first objective was to develop an organoleptically acceptable fortified rice to address nutrient deficiencies in a developing country where residents consume low amounts of iron but high amounts of rice. The second objective of this project was to understand the effects of tea's iron inhibitory compounds on the body. The following sections in this chapter review Food Fortification with Iron, Tea Production & Chemistry and Proline Rich Proteins in the Saliva.

1.2 Food Fortification with Iron

The most cost-effective and universally applicable strategy in the prevention of iron deficiency is through the implementation of food fortification programs (Hurrell 1997). In both developed and developing countries, fortification of staple foods has led to decreased incidence of iron deficiency anemia and improved iron status (Yip *et al.* 1987; Walter *et al.* 1993). The successful fortification of a food product requires that the food vehicle used is commonly consumed by the target population and that the food vehicle is matched to the appropriate iron compound. Fortifying a staple crop ensures maximum reachability and high consumption of the fortified vehicle, as well as allowing the population to consume

their normal diet. The most frequently utilized food fortification vehicles are cereal flours, especially corn and wheat, because they are staple foods in both developing and developed countries (INACG 1982). The second factor to consider in food fortification is choosing the appropriate iron fortificant. The body is not able to utilize all forms of iron equally, and thus the degree to which the body can absorb dietary iron is characterized as iron bioavailability. While some iron compounds are more bioavailable than others, the presence of dietary inhibitors in the target food vehicle may necessitate the use of one compound over another. Additionally, a less bioavailable iron compound might be used to prevent unwanted organoleptic changes to the food vehicle's appearance, taste or smell. Here, I will review the iron compounds or powders most commonly used for fortification.

1.2.1. Commonly Used Iron Fortificants

Iron fortificants can be classified into four major groups, 1) freely water soluble compounds, 2) poorly water soluble compounds that will dissolve in dilute stomach acid, 3) water-insoluble compounds that do not dissolve well in dilute acid, and 4) chelated compounds. In general, the water soluble and the acid soluble compounds are much more bioavailable than the water-insoluble compounds because solubility in the gastric juices greatly affects the absorption of fortified iron (Hurrell 1997).

Freely Water Soluble Compounds. Due to their high solubility, these compounds have high bioavailability, which makes them the first choice for iron fortification. However, they are also highly reactive, catalyzing lipid oxidation and vitamin degradation in the fortified food. This often results in unacceptable sensory changes and reduced nutritional

quality (Hurrell 1997). These compounds are often used to fortify solid foods, like powdered infant formulas, but are generally not recommended for liquid foods (Hurrell & Cook 1990). They cannot be used in milk or cereals because of flavor changes due to lipid oxidation. However, they can be used in liquid milk-based infant formulas because these formulas are retorted and sealed so that the product remains in an anaerobic environment, which prevents oxidation. Ferrous sulfate, ferrous lactate, ferric gluconate and ferric ammonium citrate are commonly used freely water soluble iron compounds.

Ferrous sulfate is notable for both its high bioavailability and inexpensive cost. Anhydrous ferrous sulfate is a light tan powder that does not add color to fortified foods. In the past, it has been used to extensively to fortify powdered infant formulas because powdered milk fortified with ferrous sulfate has been shown to be quite acceptable by infants (Stekel *et al.* 1988). Ferrous sulfate can also be used effectively in a number of other products including soy sauce (Watanapaisantrakul *et al.* 2006), whole wheat flour (Rehman *et al.* 2006), and maize meal (Bovell-Benjamin *et al.* 1999). In these systems, the fortified food or dishes made from the fortified food showed comparable color and overall acceptability to the unfortified product or dishes made from the unfortified food. While there are applications for the use of ferrous sulfate, there are technical difficulties when using ferrous sulfate as a food fortificant, because of the pro-oxidant nature of the compound.

During storage, ferrous sulfate reacts easily with lipids in the food producing rancidity and undesirable flavors and color changes. For example, after whole wheat flour had been fortified and stored, it produced naan that showed a significant decrease in quality, based on 7 attributes including color, taste and overall acceptability (Rehman *et al.* 2006). Ferrous

sulfate has also been shown to discolor fortified extruded rice grains, turning them dark brown after just two weeks of storage (Moretti *et al.* 2005). Ferrous sulfate-fortified milk becomes oxidized and unacceptable after two day storage (Wang & King 1973). Although ferrous sulfate is commonly used in infant formula with good results, infant cereals fortified with ferrous sulfate have a tendency to turn grey or green and if the formula contains bananas, blue (Hurrell 1984). Sugar can develop a purple color if mixed with ferrous sulfate, forming a dark black discoloration when added to coffee or tea (Disler *et al.* 1975a). Morales et al (2008) found that when ferrous sulfate is added to both a supplemental powdered beverage and a baby food, the ferrous sulfate-fortified products received the least acceptance, from toddlers and their mothers, when compared to reduced iron or ferrous fumarate fortified products. The addition of ferrous sulfate to instant noodle dough decreased the color quality of both the raw dough and of the final noodles (Kongkachuichai *et al.* 2007). Mashed potatoes turn a dark gray-green color upon the addition of ferrous sulfate (Sapers *et al.* 1974).

Ferrous lactate and ferrous gluconate are freely water soluble compounds that have limited applications because they are more costly than ferrous sulfate with many of ferrous sulfate's organoleptic problems. Ferrous lactate has been used mostly in milk and soy-based formulas (Cook & Reusser 1983). Watanapaisantrakul *et al* (2006) found that either ferrous gluconate or ferrous lactate could be added to soy sauce without causing a deterioration of product quality. However, chocolate milk fortified with ferrous lactate develops a significant discoloration (Douglas *et al.* 1981). Kiskini *et al* (2007) found that when ferrous lactate was used to fortify soy flour, the resulting gluten-free bread received scores equaling a totally

unacceptable product; when ferrous gluconate was used to fortify the flour, the resulting bread received a score statistically similar to the unfortified gluten-free bread.

Ferric ammonium citrate has been used in the UK in the fortification of flour and has a bioavailability similar to other water soluble iron fortificants (Cook & Reusser 1983; Hurrell 1997). Ferric ammonium citrate discolors mashed potatoes (Sapers *et al.* 1974) but does not destroy soy sauce quality, even after three months storage (Watanapaisantrakul *et al.* 2006). Milk fortified with ferric ammonium citrate was judged acceptable and shown to be similar to the unfortified milk (Wang & King 1973). However, the addition of ferric ammonium citrate to chocolate milk caused noticeable darkening within fourteen days (Stekel *et al.* 1988).

Poorly Water Soluble/Acid Soluble Compounds. These compounds are used in fortification because they are less reactive in the food product than water soluble compounds. Additionally because their acid solubility allows them to be soluble in gastric juices, they have good bioavailabilities. However, they cannot be used to fortify flour because, during long term storage, these compounds promote rancidity (Salgueiro *et al.* 2002). These compounds also tend to precipitate when added to liquids (Hurrell & Cook 1990).

Ferrous fumarate is an acid soluble iron fortificant that has a similar bioavailability to ferrous sulfate, but has fewer negative organoleptic effects compared to ferrous sulfate (van Stuijvenberg *et al.* 2008). Ferrous fumarate can be used to fortify infant cereals because it does not provoke discoloration or rancidity during six-month storage (Hurrell *et al.* 1989). The addition of hot water and hot milk to the stored, fortified infant cereal resulted in a meal

that was still acceptable. Ferrous fumarate has also been found acceptable for use in a flavored powdered baby food and beverage, producing an acceptable reconstituted product even after 6 months of dry powder storage (Morales *et al.* 2008). Ferrous fumarate is not without fortification difficulties, as it causes immediate and unacceptable precipitation in four types of soy sauce when used as the iron fortificant (Watanapaisantrakul *et al.* 2006).

Water Insoluble/Poorly Acid Soluble Compounds. These compounds are the most highly utilized in food fortification programs because they do not react with other components in the fortified product. Water insoluble compounds tend to have lower bioavailability due to their poor solubility in gastric juices, however, they can be used to fortify many food products without concern for rancidity or color changes after long term storage (Salgueiro *et al.* 2002). As such, they are often used to fortify cereals and flours, but because the bioavailability is significantly lower, it is often recommended that they are fortified at double the target amount of iron (WHO/FAO 2006).

Elemental iron powders (reduced, electrolytic and carbonyl) are insoluble in water, poorly soluble in acid and very stable when used to fortify food vehicles. The stability of all elemental irons helps protect against color and off-flavor development during food product storage. For this reason, elemental iron powders are commonly added to cereal products and complementary foods (Akhtar & Anjum 2007). Morales et al (2008) found that when reduced iron is added to a supplemental powdered baby food and beverage, the reduced iron-fortified products were acceptable and well received. Unfortunately, the slight metallic taste and gray color of iron fortificants themselves can sometimes be tasted and seen in the product. When Akhtar & Anjum (2007) added elemental iron to whole wheat flour at 40

ppm, the fortified flour imparted a blackish discoloration to the naan. When elemental iron was used to fortify soy flour, the resulting gluten-free bread was judged unacceptable by panelists (Kiskini *et al.* 2007). The addition of elemental iron powder to a rice premix, resulted in extruded rice grains that were noticeably white-gray in appearance (Moretti *et al.* 2005). Additionally, elemental iron compounds have poor bioavailability due to their low solubility in gastric juices. They have been shown to have absorptions less than half that of ferrous sulfate (Hoppe *et al.* 2006). It is recommended that only electrolytic iron powder is used for fortification and that it should be used at twice the target concentration to account for the decreased bioavailability (Hurrell *et al.* 2010).

Ferric pyrophosphate is poorly soluble in acid and has a low bioavailability. It was estimated that adult human absorption of ferric pyrophosphate from a fortified cereal is only half of the absorption from ferrous sulfate (Hurrell *et al.* 1989). However, micronized ferric pyrophosphate has been shown to have good bioavailability (Zimmermann *et al.* 2004; Wegmuller *et al.* 2006). When compared to regular ferric pyrophosphate (mean particle size, 8 microns), micronized ferric pyrophosphate (mean particle size, 0.5 microns) has 2 to 4 times greater iron absorption from milk (Fidler *et al.* 2004). However, ferric pyrophosphate is useful for food fortification because it produces a highly acceptable product that remains acceptable even after long-term storage.

Ferric pyrophosphate-fortified pre-cooked whole wheat flour (15 mg iron per 100 g flour), was judged acceptable after 7 weeks of storage (Hurrell 1997). Ferric pyrophosphate, added to rice cereal along with sodium citrate or ammonium citrate, produced low amounts of oxidation, not different from the unfortified rice cereal (Hurrell *et al.* 1989). Gluten-free

bread with added ferric pyrophosphate had a similar taste and overall quality compared to the unfortified gluten-free bread (Kiskini *et al.* 2007). Douglas et al (1981) found ferric pyrophosphate acceptable for the fortification of chocolate milk. Moretti et al (2005) demonstrated that when micronized ferric pyrophosphate is added to powdered rice mix, the extruded rice color was similar to the natural rice grain.

Chelated-Iron Compounds. Iron chelates show much promise as iron fortificants because they demonstrate high bioavailability from foods, even in the presence of iron inhibitors (Salgueiro *et al.* 2002). For these compounds, cost is a major deterrent from their use, as they are considerably more expensive than other iron compounds (WHO/FAO 2006).

Sodium ethylene diamine tetraacetic acid (NaEDTA) is a chelator that protects and prevents the iron from interacting with iron inhibitors, such as phytic acid. Considering that cereals and legume meals can contain high amounts of phytic acid, it has been shown that using iron NaEDTA (NaFeEDTA) in fortification can increase iron bioavailability from a high phytate meal, 2 to three times higher than the bioavailability of ferrous sulfate from the same meal (Hurrell 1997). The chelation effect also can prevent the iron from acting as a catalyst in oxidation reactions during storage within a fortified food vehicle. After a twenty day storage, whole maize fortified with NaFeEDTA showed only low amounts of oxidation, and the porridge made from the NaFeEDTA-fortified maize received acceptable color and rancidity scores (Bovell-Benjamin *et al.* 1999). Product quality of raw noodle dough and formed instant noodles was not changed by the addition of NaFeEDTA, and even after three month storage at room temperature, product quality of the instant noodles was not

changed by fortification (Kongkachuichai *et al.* 2007). Another study performed on noodles demonstrated that adults and children could discriminate between NaFeEDTA-fortified noodles and the unfortified noodles, but showed no preference for either, indicating that acceptance was high for the fortified noodles (Le *et al.* 2007). NaFeEDTA can be used to fortify soy sauce (Watanapaisantrakul *et al.* 2006) and fish sauce (Garby & Areekul 1974) as it will not cause unacceptable precipitation in either condiment. NaFeEDTA has also been used successfully in the fortification of various wheat products. The fortification of wheat biscuits using NaFeEDTA creates a final product that is organoleptically similar to unfortified biscuits (Govindaraj *et al.* 2007). Kiskini et al (2007) have found that NaFeEDTA fortified soy flour produces an acceptable gluten-free bread. When NaFeEDTA-fortified whole wheat flour is baked into chapattis (an unleavened bread made of flour and water), the NaFeEDTA fortificant contributes less color to the chapattis as compared to elemental iron (Akhtar & Anjum 2007).

However, NaFeEDTA is not without fortification challenges. Sugar fortification is possible using NaFeEDTA, but the resulting sugar develops a yellow tint (Viteri *et al.* 1995). When the fortified sugar was used to sweeten tea or coffee, the beverage noticeably darkened. All baked goods made from the fortified sugar had some dark color development, but were still acceptable. Curry powder with added NaFeEDTA will darken products, but not to the extent of rejection (Lamparelli *et al.* 1987). When NaFeEDTA was added to rice flour mix, the extruded grains turned brown-reddish after two weeks of storage (Moretti *et al.* 2005).

Glycinate is an amino acid chelator that prevents iron from interacting with phytic acid in the meal. Ferrous bisglycinate has been shown to have a high bioavailability from a fortified maize meal, but was highly unstable during storage of the fortified whole maize meal, producing significant rancidity (Bovell-Benjamin *et al.* 1999). Ferrous bisglycinate is also unstable in a fortified soy sauce, causing immediate precipitation (Watanapaisantrakul *et al.* 2006).

Characteristics of the iron compounds described above are presented in Table 1-1.

1.2.2. Food Fortification Programs

While there are few studies evaluating the effectiveness of food fortification programs, fortification of staple foods is believed to have significantly affected the prevalence of anemia in both developed and developing countries. In the United States, iron fortification of infant formulas and cereals was associated with a decrease in anemia prevalence in children under 5 years old (Yip *et al.* 1987; Fomon 2001). In Venezula, which began fortifying wheat and maize flours in 1993, the fortification program has been associated with reduced prevalence of iron deficiency and anemia in Venezuelan children (Layrisse *et al.* 1996). In Chile, fortification of milk with iron and ascorbic acid is associated with decreased prevalence of iron deficiency in infants and young children (Stekel *et al.* 1988; Hertrampf 2002). A two-year study of 10,000 Chinese women demonstrated that fortification of soy sauce with iron has the potential to be an effective fortification program in China and other countries where soy sauce is a staple condiment (WHO/FAO 2006).

Table 1-1. Key characteristics of iron compounds used to fortify food (adapted from *WHO 2006*)

Compound	Iron Content (%)	Relative bioavailability	Relative Cost (per mg iron)	Recommended Fortification Vehicles
Water soluble				
Ferrous sulfate, dried	33	100	1.0	Low extraction wheat flour (white), degermed corn flour, pasta, dry milk, soy sauce, infant cereal
Ferrous gluconate	12	89	6.7	
Ferrous lactate	19	67	7.5	
Ferric ammonium citrate	17	51	4.4	Fluid milk
Poorly water soluble, soluble in dilute acid				
Ferrous fumarate	33	100	2.2	White flour, degermed corn flour, infant cereal; Whole wheat, corn flour
Water insoluble/poorly soluble in acid				
Ferric pyrophosphate	25	21-74	4.7	Rice, fluid milk, juice, soft drinks
Elemental:				
Hydrogen reduced	96	13-148[a]	0.5	
Carbonyl	99	5-20	2.2	
Electrolytic	97	75	0.8	White flour, degermed corn flour, infant cereals, breakfast cereals
Chelated Iron[b]				
Ferrous bisglycinate	20	100	17.6	
Sodium iron EDTA	13	100	16.7	Whole wheat flour, corn flour, soy sauce, sugar

[a]The high upper limit is from a study that utilized a very small particle size; one that is considerably smaller than any used in fortification programs.
[b]Absorption is 2 or 3 times higher than ferrous sulfate if the food is high in phytate.

1.3 Tea Production & Chemistry

All varieties of tea are produced from the species *Camellia sinensis* (L.) O. Kuntze, a perennial leafy plant that grows in warm, humid climates. There are two varieties: var. *sinensis*, which has small leaves (5-12 cm) and var. *assamica*, which has longer leaves (up to 20 cm)(Graham 1992). The conditions applied prior to and during the drying process of the leaves determine the type of tea. The three basic types of tea are green tea, black tea and oolong tea and they are produced by either preventing or encouraging oxidation of the leaves. To produce green tea, the leaves are heated in a manner that deactivates the natural polyphenol oxidase, thereby preventing oxidation of polyphenols in the leaves. This is in contrast to black tea production where enzymatic oxidation of catechins is key to obtaining the characteristic flavor and dark color. Partial oxidation of the tea leaves results in oolong tea.

Fresh tea leaves are plucked either by hand or machine, with the highest quality teas always starting with hand plucked leaves. The leaves are immediately transferred to tea factories for processing. Upon arrival, leaves destined for green tea are fixed by pan-firing or steaming to prevent oxidation (Cabrera *et al.* 2006). Typically, pan-fired teas are produced in China, while Japanese teas are typically steamed (Wan *et al.* 2009b). After fixing, the leaves are rolled and dried prior to finishing. The finishing step determines the type of green tea produced and involves removal of impurities, cutting, grading, blending and packing. The finished product usually appears uniform and characteristic of the type of tea; for example Gunpowder tea should appear as small, tight round balls, whereas Dragonwell tea has pieces with a characteristic long, flat shape.

Fresh leaves destined for black tea are first withered and rolled, to promote release of the leaves' natural polyphenol oxidase. The leaves are transferred to a warm (25-35°C), humid (>95% humidity) room for fermentation, which can last from 30 minutes to 3 hours (Wan *et al.* 2009b). Fermentation time is dependent on the age of the leaves, fermentation conditions and the manner in which the leaves were rolled. After the leaves are optimally fermented, the leaves are heated to arrest further oxidation (Cabrera *et al.* 2006). Similar to green tea, after drying the leaves are sent for finishing and gradating. These conditions of black tea production promote the oxidation and polymerization of catechins and their reaction products, resulting in black tea's characteristic color and flavor.

Oolong tea is a partially fermented tea that combines the green freshness of green tea with the earthy aroma of black tea. Upon arrival at the tea factory, leaves destined for oolong tea are shaken to bruise the edges of the leaves and then allowed to undergo partial fermentation (Wan *et al.* 2009b). After fermentation, the leaves are heated to stop oxidation and sent for finishing.

1.3.1 Major Components of Green Tea

Tea infusions prepared from green tea leaves are often light in color, as the leaves have not undergone significant enzymatic oxidation. Green tea infusions, similar to the leaves, are high in catechins, which are colorless, water-soluble and readily oxidizable flavonoids. Catechins are important to green tea in that they provide astringency and bitterness (Wang *et al.* 2000). It has been reported that tea catechins comprise 30-42% of the dry weight of green tea infusions (Dufresne & Farnworth 2001). The most notable catechins

are epigallocatechin gallate (EGCG), epigallocatechin (EGC), and epicatechin gallate (ECG), with EGCG by far the most abundant. A typical cup (237 ml) of green tea contains 30 to 130 mg of EGCG (Balentine & Paetau-Robinson 2000). Chemical structures are shown in Figure 1-1. Relative percentages of catechins from a fresh tea leaf are shown in Table 1-2.

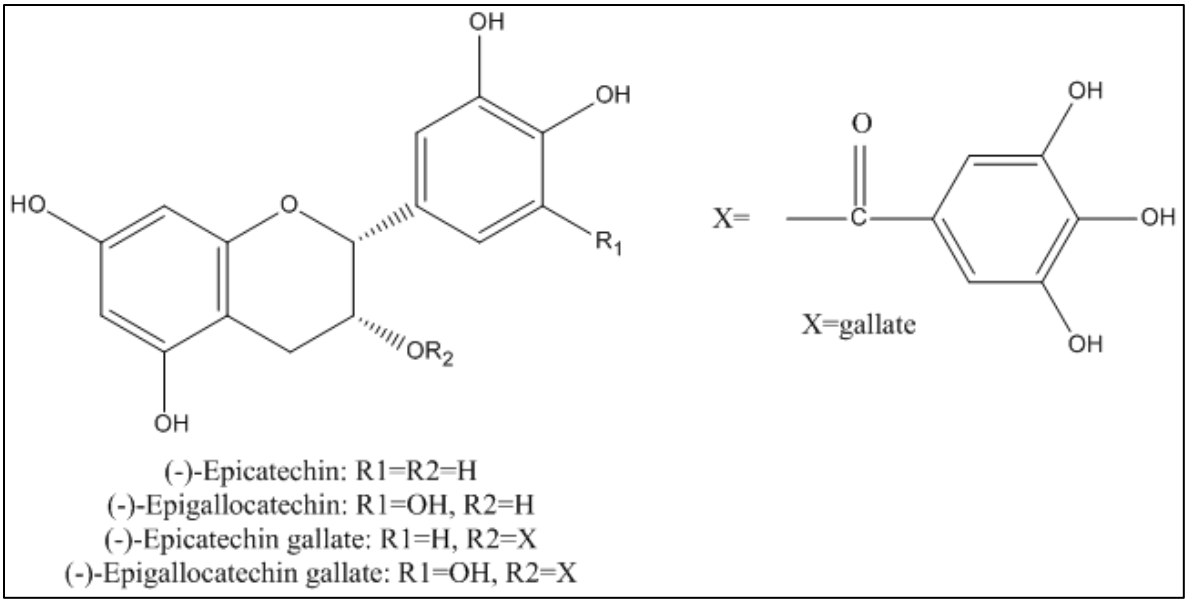


Figure 1-1: Tea catechin structures (redrawn from Wang *et al.* 2000)

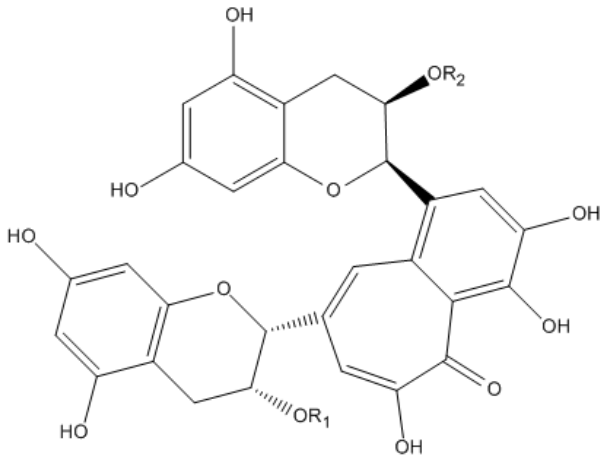
Table 1-2: Catechins in tea leaves (adapted from Wan *et al.* 2009b)

Catechin	**Content (dry weight)**
(—)-Epicatechin (EC)	1-3%
(—)-Epigallocatechin (EGC)	3-6%
(—)-Epicatechin gallate (ECG)	3-6%
(—)-Epigallocatechin gallate (EGCG)	8-12%
(+)-Catechin (C)	1-2%
(+)-Gallocatechin (GC)	3-4%

1.3.2 Major Components in Black Tea

Tea infusions prepared from black tea leaves are dark in color due to the thorough oxidation of the catechins in the leaves. The resulting tea is much lower in catechins (10-12%, dry weight), but contains new components such as theaflavins and thearubigins, which together create black tea's characteristic dark color (Luczaj & Skrzydlewska 2005). Both also contribute to the *creaming* of black tea, in which caffeine forms an insoluble complex with theaflavins and thearubigins, to a seven times greater extent with thearubigins (Powell *et al.* 1993). This process occurs upon the cooling of a strong brew and tea tasters use the appearance and quantity of the cream to estimate tea quality (Roberts 1963; Cloughley 1981).

Theaflavins are orange-colored pigments that comprise 0.3-2% of black tea leaves, dry weight basis, and 3-6% of black tea infusion (Graham 1992; Wang *et al.* 2000; Haslam 2003). The major theaflavins found in black tea are theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate and theaflavin 3,3'-gallate and they are the products of oxidative coupling reactions between pyrogallol-type catechins (EGC and EGCG) and catechol-type catechins (EC and ECG) (Wang *et al.* 2000; Tanaka *et al.* 2009). The structures of theaflavins can be found in Figure 1-2 and their relative concentrations within a cup of black tea can be found in Table 1-3.



Theaflavin: R1=R2=H
Theaflavin 3-gallate: R1=gallate, R2=H
Theaflavin 3'-gallate: R1=H, R2=gallate
Theaflavin 3,3'-gallate: R1=R2=gallate

Figure 1-2: Theaflavin structures (redrawn from Wang *et al.* 2000)

Table 1-3: Theaflavins found in black tea (adapted from Wan *et al.* 2009b)

Precursors	**Theaflavins**	**Content (dry weight)**
EC + EGC	Theaflavin	0.2-0.3%
EC + EGCG	Theaflavin 3-gallate	1.0-1.5%
ECG + EGC	Theaflavin 3'-gallate	
ECG + EGCG	Theaflavin 3,3'-gallate	0.6-1.2%

Thearubigins are a large class of ill-defined water-soluble pigments that comprise 10-20% of black tea infusion, dry weight (Haslam 2003; Luczaj & Skrzydlewska 2005). They have molecular masses reported to range between 700-40,000 Da (Wang *et al.* 2000). A subclass of thearubigins has been identified as theasinensins. These compounds are produced by oxidative coupling between two B-rings of pyrogallol-type catechins (Tanaka *et al.* 2002; Tanaka *et al.* 2009). The chemical structures of thearubigins and theasinensins can be found in Figures 1-3 and 1-4 respectively.

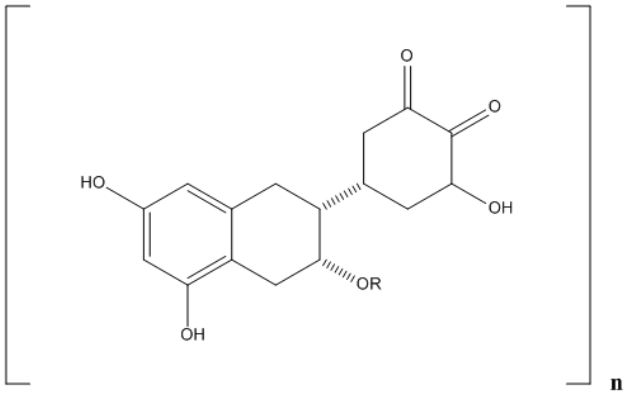


Figure 1-3: Thearubigin general structure (redrawn from Wang *et al.* 2000)

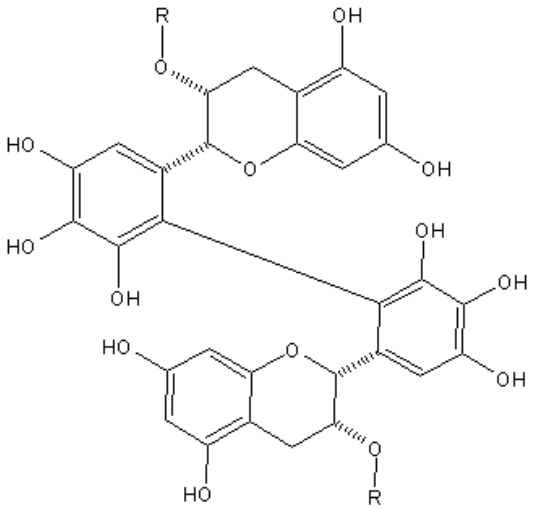


Figure 1-4: Theasinensin general structure (redrawn from Tanaka *et al.* 2002)

1.3.3 Minor Components of Green & Black Tea

Carbohydrates. Similar to the leaves of other green plants, fresh tea leaves contain carbohydrates. There is a wide range of carbohydrates reported, ranging from simple sugars, such as sucrose, glucose and fructose, to complex polysaccharides, such as cellulose, a major component of cell walls.

Proteins & amino acids. Proteins, which make up an important fraction, compose a considerable percentage of the fresh tea leaves, but do not compose a significant percentage of tea infusion solids. Amino acids identified in tea leaves and infusions include aspartic acid, arginine, glutamic acid, glycine, lysine, leucine, threonine, tyrosine, tryptophan, serine and valine (Chacko *et al.* 2010). Amino acids in tea can contribute to improved flavor by undergoing oxidation during fermentation resulting in aldehydes that can contribute to tea aroma (Perera & Wickremasinghe 1972).

Other Phenolic Compounds. Tea contains many phenolic compounds, including methyxanthines and flavonols. Of the methylxanthine component, caffeine (1,3,7-trimethyl xanthine) is a major contributor to tea quality. As mentioned above, a relatively high caffeine content in black tea is associated with higher "briskness" and "liveliness" in the tea, both characteristics of a high quality tea. Methylxanthine compounds are comparable between green and black tea infusions (Astill *et al.* 2001). Tea also contains flavonols, most notably quercetin and kaempferol. Flavonol content is relatively unaffected by processing and black tea infusions contain equivalent amounts of flavonols, as green tea infusions do (Balentine & Paetau-Robinson 2000; Dufresne & Farnworth 2001).

Minerals, pigments & volatiles. Tea contains various trace minerals and elements, including calcium, chromium, magnesium, copper, iron, zinc, selenium, sodium, potassium, aluminum, manganese, strontium, nickel, cobalt, phosphorus, and fluorine (Chacko *et al.* 2010). Pigments, such as carotenoids and chlorophyll, and volatile compounds, such as aldehydes, esters and lactones, make up a very small fraction of tea infusions but do contribute to the color and aroma of the tea.

While variety, growing environment, harvest and manufacturing conditions and infusion preparation can all affect the relative percentages in the final tea infusion, a general comparison of the principal components of tea leaves, green tea infusion and black tea infusion is presented in Table 1-4.

Table 1-4: Comparison of the Principal components of Fresh Green Tea Leaf, Green Tea Beverage, and Black Tea Beverage (compiled from Graham 1992; Chacko *et al.* 2010)

Compound	**Fresh Tea Leaf**[a]	**Green Tea Beverage**[b]	**Black Tea Beverage**[b]
Catechins	36	30-42	3-12
Methylxanthines	3.5	7-9	8-11
Caffeine		1-4	2-4
Other flavonols		5-10	6-9
Amino Acids	4	4	4
Organic Acids	1.5	5-7	10-12
Carbohydrates	25	10-15	15
Protein	15	Trace	trace
Mineral ash	5	4.5	4.5
Pigments	2	Trace	trace
Theaflavins			3-6
Thearubigins			10-20

[a]Expressed as % of leaf, dry weight. [b]Expressed as % of extract solids.

Tannins & Tea Polyphenols

Here I would like to briefly discuss tannins and, in agreement with other authors (Graham 1992; Yang & Wang 1993), propose that we refer to catechins, thearubigins, theaflavins, and other flavonoid compounds in tea, as "tea polyphenols" instead of "tea tannins".

Tannins are a group of natural plant compounds characterized by numerous phenolic groups and molecular weights larger than 500 (Mehansho *et al.* 1987b; Bennick 2002). By definition, tannins must also be able to precipitate gelatin. In industry, tannins are defined as plant compounds that produce a blue color when mixed with ferric iron and must be able to turn hide into leather. Within those definitions, there is great variability in the structures of tannins. It should be noted that while the terms "tannin" and "tannic acid" are often used interchangeably, they are not interchangeable. "Tannins" refers to a diverse group of polyphenolic compounds, including but not limited to tannic acid and its specific derivatives (Hamilton-Miller 1995). It is important to note, while some of the polyphenols in tea can be classified as tannins, the tannic acid content of tea is zero (Wheeler 1979).

Hydrolyzable tannins. Structurally, tannins are categorized as either hydrolyzable or condensed. Hydrolyzable tannins usually consist of a glucose molecule, or other polyhydric alcohol, connected to multiple gallic acid units (or derivatives) through ester linkages. These tannins can be hydrolyzed in alkaline solutions. The structure of gallic acid is shown in Figure 1-5, and pentagalloyl glucose, a hydrolyzable tannin, is depicted in Figure 1-6.

Condensed tannins. Condensed tannins are polymers of flavan-3-ols and flavan-3,4-diols connected through carbon-carbon linkages. These tannins can have molecular

weights larger than 30,000 and are highly resistant to hydrolysis. However condensed tannins can be depolymerized in hot, highly acidic solutions, resulting in anthocyanidin pigments and various other reaction products (Bennick 2002). As such, these tannins are sometimes referred to as proanthocyanidins. The generic structure for a condensed tannin is shown in Figure 1-7.

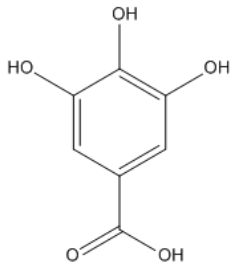


Figure 1-5: Gallic acid structure (redrawn from Bennick 2002)

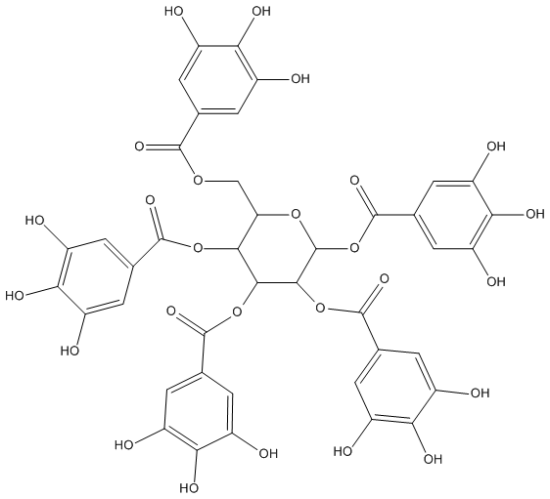


Figure 1-6: Pentagalloyl glucose, a hydrolyzable tannin (redrawn from Bennick 2002)

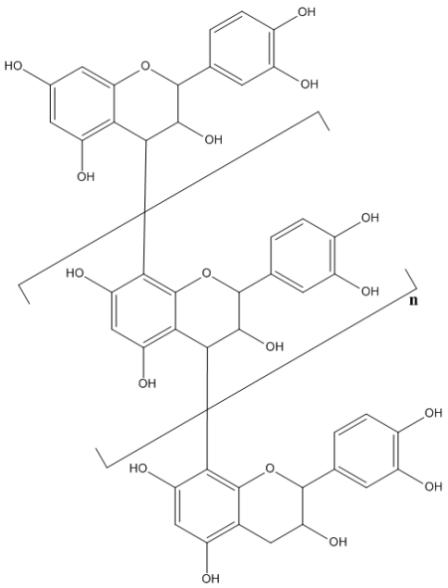


Figure 1-7: General structure of condensed tannins (redrawn from Bennick, 2002)

When added to diets, tannins have been shown to cause decreased weight gains in mice and hamsters (Mehansho *et al.* 1987b; Chung *et al.* 1998) and decreased protein digestibility and utilization in humans and rats (Eggum & Christensen 1975; Hussein & Abbas 1985). Additionally, *in vitro* studies of tannins demonstrate their ability to inhibit nearly all of the digestive enzymes, but this effect appears to have little significance *in vivo* (Chung *et al.* 1998). Instead, it is believed that tannins decrease protein digestibility by directly complexing the dietary proteins (Mitaru *et al.* 1984).

As mentioned above, several authors have proposed that use of the term "tea tannins" when referring to catechins is both confusing and misleading. Yet the term "green tea tannins" is still utilized in current publications and catechins are commonly considered to be tannins (Nakagawa & Yokozawa 2002). However, the more appropriate term for catechins is "tea polyphenols". While tea catechins can precipitate gelatin, they have

molecular weights under the 500 molecular weight cutoff of tannin classification and they cannot be used for tanning hides (Yang & Wang 1993). With respect to black tea, thearubigins and theaflavins are large molecules that could appropriately be called black tea "tannins". However, for continuity and simplicity, they will be referred to by the more general term, "polyphenols".

1.3.4 Bioavailability of Tea Polyphenols

It is believed that tea catechins are predominantly absorbed in the intestines (Okushio *et al.* 1996), however, catechins can also be absorbed in the mouth through the oral mucosa. Yang *et al.* (1999) reported that when humans subjects held an EGCG solution in their mouths, without swallowing, EGCG and EGC were found in the saliva, but only EGC was found in the urine. It was suggested that a salivary catechin esterase converts some EGCG into EGC prior to oral absorption and then excretion through urine. Yang *et al.* (1998) administered various amounts of green tea catechins to 18 participants (1.5, 3.0, and 4.5 g of decaffeinated green tea solids dissolved in 500 ml of water). For three of the principal catechins—EGCG, EGC and EC—maximum plasma concentrations were 190-550 ng/ml and these values were observed approximately 2 hours after the tea ingestion. Once absorbed, tea catechins undergo methylation, glucuronidation and sulfation to produce a wide range of metabolites, most of which are not characterized (Sun *et al.* 2009).

Unabsorbed tea catechins that reach the large intestines are broken down by bacterial enzymes into simpler compounds, which are then absorbed by the body (Li *et al.* 2000; Kohri *et al.* 2001). Four major microbial metabolites have been identified: (—)-5-(3',4',5'-

trihydroxyphenyl)-valerolactone [M4], (—)-5-(3',4'-dihydroxyphenyl)-valeroactone [M6], (—)-5-(3',5'-dihydroxyphenyl)-valerolactone [M6'] and hippuric acid. After administration of a single dose of green tea, M4 and M6, were excreted in urine at a concentration 8 to 25 times higher than EGC and EC in human subjects (Li *et al.* 2000). The combined excretion of the metabolites M4, M6 and M6' after green tea ingestion, accounts for 1.5 -16% of the ingested catechins (Meng *et al.* 2002). The metabolite associated with black tea consumption is hippuric acid, although both green and black tea consumption increases the presence of hippuric acid in the urine (Mulder *et al.* 2005).

There is evidence that after absorption, small amounts of catechins are distributed throughout the body's tissues. When rats were administered green tea polyphenols in their drinking water (0.6% w/v) for 28 days, EGC and EC were found at substantial levels in the bladder, esophagus, kidney, large intestine, lung and prostate, but were present at lower levels in the liver, spleen, heart and thyroid (Kim *et al.* 2000). EGCG levels were higher only in the esophagus and large intestine. In humans, patients who consumed tea 12 hours prior to surgery had substantial amounts of catechins present in their colon mucosa and prostates (Yang *et al.* 2000). In men consuming green or black tea for 5 days before a radical prostectomy, significant amounts of catechins were found in the prostate samples (Henning *et al.* 2006). More research is needed, in humans and animals, to understand tissue distribution of catechins and their metabolites and how this affects their biological activities.

Tea catechins and their metabolites are excreted via urine or bile. In general, EGC and EC are excreted in urine or bile, but EGCG is predominantly excreted in bile (Lee *et al.* 1995; Chen *et al.* 1997; Sun *et al.* 2009).

1.3.5 Effect of Tea on Health

Because tea is such a popular beverage, much interest has been demonstrated in studying the health benefits of both black and green tea consumption (Trevisanato & Kim 2000; Dufresne & Farnworth 2001; Chacko *et al.* 2010). In a study of 6,597 elderly French men and women (aged 65 years and older), there was a significant inverse relationship between tea drinking and prevalence of carotid plaques in women but not in men (Debette *et al.* 2008). In a follow-up study using 661 French women aged 59-72 years, the plaque frequency was 18.8% for women drinking no tea and 8.9% for women consuming 3 or more cups of tea daily (Debette *et al.* 2008). Also, in women, tea drinking has been shown to be inversely related with severe aortic atherosclerosis (Geleijnse *et al.* 1999). A U.K. study of 1,276 women (aged 65 to 76 years), found that those that did not consume tea daily had a significantly lower bone mineral density at the lumbar spine and hip compared to tea drinkers (Hegarty *et al.* 2000). This study is mirrored by Devine *et al* (2007) who found that tea drinking is significantly associated with the preservation of hip structure in older women.

A study of 8,522 Japanese men and women reported that men who consumed more than 10 cups (1500 ml) of green tea daily had a 54% decreased risk of death from coronary heart disease (CHD) compared to men who consumed less than 3 cups (450 ml) daily (Nakachi *et al.* 2000). In a 15-year study of 552 Dutch men, men who consumed more than 4.7 cups of black tea per day had a 69% decreased risk of stroke than men who drank less than 2.6 cups of black tea per day (Keli *et al.* 1996). In a randomized crossover study, participants with mildly elevated cholesterol were provided five servings of black tea or a

placebo for three weeks, along with identical meals to ensure that diet differences did not affect results (Davies *et al.* 2003). After three weeks, those consuming tea reduced their serum total cholesterol and LDL cholesterol by 3.8% and 7.5%, respectively, when compared to the placebo.

In mice, green tea polyphenols applied topically inhibited chemically-induced tumor formation, reducing the number of mice with tumors by 71% and reducing the number of tumors per mouse by 94% (Conney *et al.* 1992). When green tea was given as the sole liquid substance and the mice were exposed to UVB light, the formation of red sunburned lesions was inhibited in a dose-dependent manner.

In rats that had been injected with streptozotocin (STZ) to induce hyperglycemia, both black and green tea extracts were effective at reducing blood glucose levels compared to the animals receiving only water, although black tea was more effective (Gomes *et al.* 1995). When the rats were given tea prior to the STZ injection, green tea completely inhibited any increase in blood glucose level and black tea significantly reduced the impact of STZ, compared to the control. Therefore, tea infusions may be able to inhibit diabetes formation or help maintain normal glucose levels in those living with diabetes.

It has been reported that green tea and black tea are not interchangeable because green tea has a higher antioxidant capacity per serving; 436 mg vitamin C equivalents/200 mL for green tea versus 239 mg vitamin C equivalents/200 mL for black tea (Lee *et al.* 2002). However, while green tea has been reported to have a lower IC_{50} than black tea (0.23 μM versus 0.38 μM for green and black tea, respectively), both have values lower than concord grape juice (0.65 μM) and red wine (0.45 μM) (Vinson & Dabbagh 1998). IC_{50} indicates the

concentration of antioxidant needed to inhibit oxidation catalyzed by cupric ion by 50%; therefore the smaller the IC_{50}, the more potent the antioxidant is. This research indicates that both green and black teas are more powerful antioxidants than either red wine or grape juice.

Effects of tannin on iron absorption

In contrast, there are health concerns of tea consumption because of the high polyphenol content, which has an inhibitory effect on iron absorption. The effect of tea on iron absorption was first reported by Disler *et al.* (1975b), who found that tea inhibited iron absorption from both solutions of iron ($FeCl_3$ and $FeSO_4$) and from foods (bread and a meal of rice with potato and onion soup). The decrease in nonheme iron absorption was attributed to the formation of insoluble iron-polyphenol complexes in the gastro-intestinal tract, rendering the iron unavailable for absorption. Tea drinking did not affect the absorption of heme iron from meat. Since this pivotal study, many studies have been performed, using humans and rats, to understand the complex interactions between tea and iron absorption. Several of these studies are summarized in Table 1-5 (humans) and Table 1-6 (rats).

In rats, short term feeding trials usually indicate that iron absorption is reduced with concurrent tea consumption. However, conflicting data appear when the experimental period is longer than 7 days. Rats fed a diet with 1.17% (wt/wt) tea extract added to their diet for 14 days did not show any differences in weight or iron metabolism compared to the

Table 1-5: Effects of tea on iron absorption (human studies)

Author	Age of subjects	Fe given	Tea level	Length of Experiment	Results
Disler *et al.* (1975b)	26-60 y	30 μg ^{59}Fe or ^{55}Fe	200 ml tea (5 g dry tea leaves)	Single meal	Reduced absorption to 29% of control
Morck *et al.* (1983)	18-50 y	3 mg plus tracer: ^{59}Fe or ^{55}Fe	200 ml tea (1.75 g dry tea leaves)	Single meal	Reduced nonheme iron absorption to 36% of control
Wang and Kies (1991)	21-27 y	15 mg/day	8 g instant tea/day	14 days	Hb and TIBC, NSD; Reduced SI conc, 95% of control
Reddy and Cook (1991)	18-40 y	4.1 mg	200 ml tea (1.75 g dry tea leaves)	Single meal	Reduced absorption to 17% of control
	18-40 y	4.1 mg	200 ml tea (5.25 g dry tea leaves)	Single meal	Reduced absorption to 9% of control
Hurrell *et al.* (1999)	19-40 y	100 μg ^{59}Fe or ^{55}Fe	300 ml tea (3 g dry tea leaves)	Single meal	Reduced absorption by 79-94%
Samman *et al.* (2001)	19-39 y	77 μg ^{59}Fe or ^{55}Fe	117 mg green tea polyphenols	Single meal	Reduced absorption to 73% of control
Thankachan *et al.* (2008)	18-35 y	3 mg ^{57}Fe or ^{58}Fe	150 or 300 ml black tea (1.5 or 3 g dry tea leaves)	Single meal	Reduced absorption by 49% (1 cup tea) or 66% (2 cups tea)
	18-35 y w/IDA	3 mg ^{57}Fe or ^{58}Fe	150 or 300 ml black tea (1.5 or 3 g dry tea leaves)	Single meal	Reduced absorption by 59% (1 cup tea) or 67% (2 cups tea)

Abbreviations: NA=information not available, NSD=no significant difference, SI conc=Serum iron concentration, IDA=w/Iron Deficieny Anemia;

Table 1-6: Effects of tea on iron absorption (rat studies)

Author	Age of subjects	Fe given	Tea Level	Length of Experiment	Results
Disler *et al.* (1975c)	150-200 g rats	12.5 ppm	1 ml tea	Single dose by stomach	Reduced absorption to 51% of control
(Zhang *et al.* 1988)	Weanling rats, anemic	30 ppm	Unlimited tea infusion as sole liquid	10 days	Hb, NSD
	250 g rats	1 μCi ^{59}Fe	1 ml tea	Single dose by stomach	Reduced absorption to 58% of control
Greger and Lyle (1988)	Weanling rats, anemic	50 ppm	Diet w/2.31% (wt) green or black tea solids	23 days	% absorption, NSD
	Weanling rats	34 ppm	Diet w/3.50% (wt) instant tea	14 days	Final body weight 20% lower than control
	Weanling rats	34 ppm	Diet w/1.17% (wt) instant tea	14 days	Final body weight, NSD
Fairweather-Tait *et al.* (1991)	Weanling rats	38 ppm	Unlimited tea infusion as sole liquid: 20 g tea leaves in 1L water	4 weeks	Hb, liver Fe reduced to 93% of control
Reddy and Cook (1991)	125-150 g rats	50 ppm	Daily tea infusion: 1.75 g tea leaves in 200 ml water	> 7 days	% absorption, NSD
	125-150 g rats	50 ppm	Daily tea infusion: 5.25 g tea leaves in 200 ml water	> 7 days	Reduced absorption to 71% of control
Hamdaoui *et al.* (2003)	160-180 g rats, anemic	30 ppm	Unlimited tea decoction as sole liquid: 100 g green or black tea leaves in 1L water	14 days	Reduced Fe bioavailability from meal by 19.6 % (black tea) and 14.9% (green tea)
Hamdaoui *et al.* (2005)	NA	50 ppm	Unlimited tea decoction as sole liquid: 100 g green tea leaves in 1 L water	6 weeks	Reduced serum iron by 26%; Reduced liver storage iron by 32%

Abbrieviations: NA=information not available, NSD=no significant differences

control group (Greger & Lyle 1988). The authors estimated that the level of tea consumed by the rat diet was similar to a human consuming 10 cups of tea daily. Those authors also report that when anemic rats were fed a diet containing 2.31% green or black tea solids, there was no significant difference in iron absorption between the rats fed the tea diet and the control. Zhang *et al.* (1988) also found that when weanling rats were fed tea as their sole liquid for 10 days, iron absorption was not significantly different in the tea-fed animals versus the control. This is contrasted by a study in which rats fed a green or black tea decoction as their sole liquid for 14 days showed decreased iron absorption from their test meal (Hamdaoui *et al.* 2003). In a follow-up study, rats fed a green tea decoction for 6 weeks showed significant reductions in serum iron, as well as storage iron in the liver, spleen and femur (Hamdaoui *et al.* 2005).

The discrepancies between these findings could be due to the use of a "decoction" versus an "infusion". A typical tea infusion is made by adding 2 grams of tea per 100 ml of boiling water and allowing the mixture to steep for 4-6 minutes (ISO 2002). However, a typical tea decoction is made by adding 10 grams of tea per 100 ml of water and then boiling the solution for 20 minutes (Hamdaoui *et al.* 2003). The 5-fold higher amount of tea leaves used and the increased extraction time result in a brew considerably higher in polyphenols and therefore more likely to affect the iron status, even after 7 days of exposure. This could explain why Hamdaoui *et al.* observed that tea's iron absorption inhibitory effects did not disappear after long-term tea exposure, while older studies showed the effects did disappear.

A recent study, demonstrated that the rats adaptation to tea occurs within the first 5 days of tea exposure (Kim & Miller 2005). When rats were given test solutions of $^{59}FeCl_3$ in

tea, iron absorption was significantly inhibited. However, when rats were fed a tea diet for 5 days prior to receiving the test solution, there was no significant difference in iron absorption between the tea group and the control. Viewed together, these studies suggest that after 5-7 days of exposure to tea, rats are able to overcome the inhibitory effect of tea on iron absorption.

In humans, nearly all short term studies demonstrate that tea decreases iron absorption from a meal and can negatively affect iron status. However, most of the human studies that have been performed and all of the studies presented in Table 1-5, utilize extrinsically labeled, single test meals. Traditionally for this type of test, subjects consume a labeled test meal with tea and on the preceding or following day, the same subjects consume the same meal without tea. To differentiate between the two meals, isotopes are usually used in pairs (for example, ^{59}Fe could be used to label the test meal without tea and ^{55}Fe could be used to label the test meal with tea). After 1-2 weeks, blood is collected and analyzed for hemoglobin incorporation of the stable or radioactive isotope tracer. This method is an accurate way of measuring iron absorption from a meal, but it does not allow for the researcher to observe any adaptation effects in response to tea feeding. Perhaps this is why single test meal studies repeatedly show that tea inhibits iron absorption, but epidemiological studies do not find correlations between tea consumption and iron status.

Temme & Van Hoydonck (2002) and Nelson & Poulter (2004) reported data from 16 and 12 observational studies, respectively, both finding that after accounting for other dietary components, there was no apparent association between tea drinking and iron status or iron deficiency. In an analysis of data from the NHANES II (United States), daily tea and

coffee consumption, where the average was 3.7 cups, was negatively associated with the prevalence of anemia (Mehta *et al.* 1992). In a study of French adults, the authors reported that serum ferritin concentrations were not correlated with drinking herbal, green or black tea, in men, pre- or postmenopausal women (Mennen *et al.* 2007). Schlesier *et al.* (2012) explored the effect of tea consumption on the iron status of vegetarians and found that iron status was not affected by tea consumption. Vegetarians are a population at risk for iron deficiency due to low intake of bioavailable iron, but consumption of either green or black tea (1 liter/d) for four weeks did not affect hemoglobin, transferrin, total iron or iron binding capacity.

A majority of the focus for these epidemiological studies is on Western countries, however, an analysis of a THUSA study (South Africa), did not demonstrate consistent associations between iron status and black tea consumption in a population considered at greater risk for iron deficiency (Hogenkamp *et al.* 2008). When 150 rural black African children (aged 6-15) were given 400 mL of Rooibos or black tea daily for 16 weeks, there were no significant differences in iron status changes between the two groups (Breet *et al.* 2005). Rooibos infusions (or red bush tea) are prepared by soaking the oxidized leaves of the rooibos plant (*Aspalathus linearis*) in hot water, similar to tea steeping. Rooibos is enjoyed similarly to black tea, with milk and or sugar, but does not cause a decrease in iron absorption from a single meal (Breet *et al.* 2005).

Taken together, all of these studies seem to suggest that both rats and humans have the ability to overcome teas' deleterious effect on iron absorption. It has been suggested that increased salivary proline-rich protein secretion could explain the recovery from the

initial iron-inhibiting effects of tea (Mehansho *et al.* 1983; Mehansho *et al.* 1987b; Kim & Miller 2005).

1.4 Proline-Rich Proteins in the Saliva

1.4.1 Components of Whole Saliva

Whole saliva is considered to be the sum of secretions from three major glands—parotid, submandibular and sublingual—as well as several minor glands. Saliva is a hypotonic aqueous solution which lubricates the mouth, buffers pH in the oral cavity and maintains oral health. Lubricating glycoproteins, such as mucins, prevent damage to oral tissues from desiccation or other physical injury (Amado *et al.* 2005). Calcium and phosphorus ions promote healthy tooth enamel, while bicarbonate ions provide buffering capabilities (Dodds *et al.* 2005). Over 300 proteins have been identified in saliva's complex matrix, varying with the individual, health and diet (Amado *et al.* 2005). These proteins may be classified into several protein families that are consistently present. Proline-rich proteins constitute approximately 70% (by weight) of the parotid gland protein secretion and 40% of whole saliva proteins (by weight), in humans (Hay *et al.* 1984; Messana *et al.* 2008). Amylase is the second most abundant protein in human saliva, making up almost 30% of parotid protein secretions and 20% of whole saliva proteins (Messana *et al.* 2008). Other major proteins (by weight) in whole saliva include mucins (20%), S cystanins (8%), histanins (1%) and statherin (1%) (Messana *et al.* 2008). Whole saliva also contains lysozyme, lactoperoxidase and secretory immunoglobulin A (Yao *et al.* 2003). The human body produces between 1 to 2 liters of saliva daily (Amado *et al.* 2005).

1.4.2 Composition and Structure of Proline-Rich Proteins:

Proline-rich proteins, or PRPs, are present in the salivary glands and saliva of humans, rats, and other animals. In human whole saliva, PRPs account for more than 60% of salivary proteins, by weight (Bennick 1982; Messana *et al.* 2008). In human parotid saliva, PRPs account for more than 70% of the total protein content (Kauffman & Keller 1979). Humans secrete high levels of PRPs regardless of whether the diet is tannin-free or tannin-rich (McArthur *et al.* 1995). This is in contrast to rats and mice, who naturally secrete low amounts of PRPs but can dramatically increase their PRP secretion in the presence of dietary tannins (Mehansho *et al.* 1983; Mehansho *et al.* 1985).

Characteristically, 35-40% of amino acid residues in PRPs are proline (Bennick 2002), which is significant in that most other proteins contain no more than 5% proline (Shimada 2006). 70-88% of all amino acid residues in PRPs are either proline, glutamine, glutamic acid or glycine (Bennick 2002). It has been reported that a further 10% of residues are either lysine or arginine (Arneberg 1974). Nuclear magnetic resonance (NMR) studies indicate that PRPs have a general lack of secondary structure, instead existing as a series of randomly coiled structures (Murray & Williamson 1994; Shimada 2006). It is possible that the loose coils may allow the proteins to interact with a large variety of polyphenol structures (Hagerman & Butler 1981; Shimada 2006). Proline-rich proteins are divided broadly in three main classes, based on the isoelectric point and degree of glycosylation. The three classes are acidic PRPs, basic PRPs and glycosylated PRPs.

Basic Proline Rich Proteins (pI >7). Basic proline-rich proteins are composed mainly of four amino acids—proline, glutamine, glutamic acid and glycine—which together can make up 70 to 90% of the total amino acid content (McArthur *et al.* 1995). Proline content is generally highest in basic PRPs (30-45% of total amino acids), although some species, such as rabbits, have basic PRPs with much lower levels of proline, closer to 20% of the total amino acids (Spielman & Bennick 1989). Basic PRPs are secreted from the parotid gland, where four genes—named *PRB1*, *PRB2*, *PRB3*, and *PRB4*—code for all basic PRPs. Ten unique basic PRP species have been identified and classified as: IB1, IB4 (P-H), IB5 (P-D), IB6, IB7, IB8a, IB8b, IB8c (P-F), IB9 (P-E), II-1 and 11-2 (Kauffman *et al.* 1991; Vitorino *et al.* 2010). It is believed that these species are a result of proteolytic cleavage of precursor proteins (Madapallimattam & Bennick 1990; Bennick 2002; Vitorino *et al.* 2010).

The only known function of basic PRPs is to counteract dietary polyphenols (Lu & Bennick 1998; Shimada 2006). They have a high affinity for polyphenols and differences in sequences and sizes of basic PRPs do not affect polyphenol-binding ability (Lu & Bennick 1998). Their excellent binding ability has been attributed to the high proline and glycine content, which prevents the protein from forming folded structures and the openness of the protein allows for increased contact surface area (Jöbstl *et al.* 2004).

Acidic Proline Rich Proteins (pI<7). Acidic proline-rich proteins, like basic PRPs, have a repetitive *C*-terminal region that is composed mostly of proline, glycine, glutamic acid, and glutamine and this region structurally makes up about 70-80% of the protein (Bennick 2002; Shimada 2006). Unlike basic PRPs, acidic PRPs have an acidic *N*-terminal region, which does not contain much proline, lowering the total proline content to 15-30%.

This *N*-terminal region is similar among all acidic PRPs, differing only in the residues at positions 4 and 50, where asparagine may be substituted for aspartic acid, and position 26, where isoleucine may substitute for leucine (Azen *et al.* 1987; Madapallimattam & Bennick 1990).

Acidic PRPs are secreted from all three major salivary glands: parotid, submandibular and sublingual (Shimada 2006). They are coded for by two genes, *PRH1* and *PRH2*, which express the acidic proteins: PRP1 (proline-rich protein 1), PRP2 (proline-rich protein 2), PIFs (parotid isoelectric-focusing variant, slow), Dbs (double hand, slow) and Pa (parotid acidic protein). Four of the proteins—PRP1, PRP2, PIFs and Dbs—are proteolytically cleaved, giving rise to the smaller acidic PRPs named PRP3, PRP4, PIFf and Dbf, respectively (Robinson *et al.* 1989). The cleaved *C*-terminal fragment corresponds to the P-C peptide, the only basic PRP in submandibular or sublingual saliva (Robinson *et al.* 1989). The acidic PRP species can undergo additional posttranslational modification, such as cleavage, phosphorylation and glycosylation (Vitorino *et al.* 2010).

Acidic PRPs have apparent physiological functions besides counteracting the effects of dietary tannins. Acidic PRPs have been shown to both bind ionic calcium in saliva and adsorb to hydroxyapatite on the tooth surface (Bennick *et al.* 1981). These results indicate that acidic PRPs both help regulate calcium concentration in the saliva and prevent excess deposition of calcium phosphate on the tooth surface. Acidic PRPs may also have a less beneficial effect on oral health. It has also been observed that acidic PRPs can contribute to plaque formation by allowing the plaque-forming oral bacteria, *Actinomyces viscosus*, to attach to the tooth surface (Gibbons & Hay 1988). *A. viscosus* can bind to the *C*-terminal region of

acidic PRPs adsorbed to hydroxyapatite, but subsequent cleavage in the *N*-terminal region removes the ability of the microorganism to attach this way.

As for tannin-binding ability, in their native form, acidic PRPs have only a moderate tannin affinity and will not precipitate high amounts of tannin (Bennick 2002), but removal of the acidic *N*-terminal region greatly increases the affinity for tannins (Lu & Bennick 1998).

Glycosylated Proline Rich Proteins. Glycosylated proline-rich proteins, are the result of the glycosylation of threonine, serine and/or asparagine residues of basic PRPs, however, glycosylation can also occur on those residues in the *C*-terminal region of acidic PRPs (Spielman *et al.* 1991). PRPs can have a large variation in carbohydrate content, from very low to almost 40%, by weight (Andjic *et al.* 1970; Mehansho & Carlson 1983). Similar to basic and acidic PRPs, glycosylated PRPs have relatively high proline, glutamic acid, glutamine and glycine content. Glycosylated PRPs have been reported to be oral lubricants, reducing the friction of food particles on teeth and thereby reducing tooth wear (Hatton *et al.* 1985; Messana *et al.* 2008). Glycosylated PRPs can also adsorb to oral bacteria, causing agglutination, which reduces the microorganisms ability to colonize in the mouth (McArthur *et al.* 1995). It has been reported that, in their native form, glycosylated PRPs have only a moderate tannin affinity because they will not precipitate high amounts of tannin (Lu & Bennick 1998; Bennick 2002). However, Sarni-Manchado *et al.* (2008) observed that at low concentrations of tannins, glycosylated PRPs will not precipitate much tannin because they form protein-tannin complexes that remain soluble in saliva. These soluble protein-tannin

complexes were credited with helping to maintain lubrication of the oral cavity, leading to a reduced astringency perception (Sarni-Manchado *et al.* 2008).

1.4.3 Dietary Tannins & Proline-rich Proteins

Hagerman and Butler (1981) were the first to reveal that tannins have a high affinity for proline-rich proteins and they suggested that salivary proline-rich proteins could provide a protective effect against the anti-nutritional properties of tannins in the diet. In the first study of its kind, Mehansho *et al.* (1983) demonstrated that in response to dietary tannins, rats will increase dramatically PRP synthesis in the parotid gland. Rats were fed a diet of high-tannin sorghum and after only three days, the rats' parotid glands had enlarged 3-fold and PRP secretion had increased 12-fold. The rats fed the high-tannin sorghum lost weight at first, but on day 3, they began to gain weight. Also on day 3, the highest PRP secretion and parotid gland weight were observed. After 3 weeks of consuming the high-tannin sorghum diet, the rats given a low-tannin diet, returned their PRP secretion and parotid gland size to a normal level.

These findings clearly demonstrated that rats respond to tannins in the diet by increasing PRP secretion to decrease the toxic effects of dietary tannins. It is important to note that not all animals have the ability to increase their PRP secretion. Follow-up studies, showed that while mice have a similar ability to increase PRP secretion in response to dietary tannins (Mehansho *et al.* 1985), hamsters do not have this ability (Mehansho *et al.* 1987a). After 3 days on the high-tannin sorghum, the hamsters did not show any increase in PRP secretion, and prolonged feeding of the high-tannin diet inhibited the hamsters' growth.

McArthur *et al.* (1995) proposed that, over generations, animals with low-tannin, nitrogen-poor diets have reduced PRP secretion rates. PRPs are poorly digested, because the peptides are resistant to cleavage by digestive enzymes (Muenzer *et al.* 1979), and therefore they can act as an expensive nitrogen sink (McArthur *et al.* 1995).

Regardless of why some animals do or do not have the ability to increase PRP secretion, proline-rich proteins are an important line of defense against dietary polyphenols. Because PRPs have a high affinity for tannins, PRP-tannin complexes will form even in the presence of many other proteins with marginal or average tannin-affinity. The secretion of PRPs protects the body by complexing tannins shortly after ingestion, preventing them from inhibiting nutrient absorption and/or binding to other vital proteins in the gastrointestinal tract.

The power of PRPs to defend against dietary polyphenols was clearly demonstrated in an experiment performed by Kim and Miller (2005), using tea and gelatin as a model for PRP. Gelatin was added to act as a proxy protein for salivary PRPs because gelatin is rich in proline and has a high affinity for polyphenols. Rats were given test solutions of ^{59}Fe in water, tea or a tea + gelatin mixture. The authors found that iron absorption was decreased from the tea solution but not from the tea + gelatin solution, indicating that tea alone inhibits iron absorption but the gelatin addition prevented the tea polyphenols from inhibiting absorption. However, when rats were allowed to habituate to a tea diet, a tea + gelatin diet or the control diet for 5 days prior to administration of the test meal, there was no difference in iron absorption from the meals (Kim & Miller 2005). Significant hypertrophy of the parotid glands was observed; indicating increased PRP secretion in the

rats fed the tea diet, but not in the rats fed the tea + gelatin diet or the control diet. These findings demonstrate that proteins rich in proline counteract the inhibitory effects of dietary polyphenols on iron absorption.

THE CO-FORTIFICATION STUDIES

CHAPTER 2: DEVELOPMENT OF ACCEPTABLE FORTIFIED EXTRUDED RICE

2.1 Introduction

Meat, or muscle tissue, is an excellent source of highly available iron and zinc, but in many developing countries, the staple diets are plant-based with low or no meat intake. This results in diets with high levels of high polyphenol and phytate but low amounts of bioavailable iron and zinc. As well, the bioavailability of provitamin A compounds from dark leafy vegetables is poor compared to that from liver. As a result, deficiencies of iron, zinc and vitamin A are common in developing countries (Gibson *et al.* 2000). Women of reproductive age, infants and school aged children suffer disproportionately from iron, zinc and vitamin A deficiencies. As discussed in the previous chapter, iron deficiency has been shown to impair physical and mental development in children, increase maternal and infant mortality during childbirth and impair cognitive function in adults. Zinc deficiency is estimated to be almost as prevalent as iron deficiency with similar effects on human health, namely pregnancy complications, impaired immune response, and growth retardation (Gibson 1994). Vitamin A deficiency is the leading cause of preventable vision loss in children and the vitamin is necessary to maintain vision health, proper bone development and epithelial tissue (Wilkinson *et al.* 1981). Vitamin A deficiency affects 5 to 10 million children in developing countries, causing the death of 500,000 children each year (Murphy *et al.* 1992).

Food fortification is a cost-effective strategy for alleviating micronutrient malnutrition in a large population. An important advantage of food fortification is that

individuals at risk for iron deficiency consume increased amounts of the target nutrient without changing their normal diet (Salgueiro *et al.* 2002). The difficulty with fortification is determining the appropriate staple food for use and matching that with an appropriate fortificant, that provides good bioavailability of the target nutrient (s) without causing sensory changes. The foremost consideration of a food fortification program is that consumers may reject a fortified product if it has an off color or flavor or if it is much more expensive than the unfortified product.

Rice is an important food, consumed all around the world in industrialized and developing countries, but it is very low in iron (Hotz *et al.* 2008). In developed countries, white rice is enriched with iron but only to the level in unmilled rice (Moretti *et al.* 2005). In developing countries, the goal is to create a fortified rice that can serve to correct nutrient deficiencies in the populace. Surface coating of rice with nutrients has been attempted, but nutrient losses can occur when the consumer rinses the rice prior to cooking (Mannar & Gallego 2002). Recently, a super-fortified extruded rice, known as Ultra Rice, was created to withstand nutrient loss due to rinsing. Ultra Rice is produced by mixing high concentrations of selected micronutrients with rice flour and uses a cold extrusion process to manufacture simulated rice grains (Hotz *et al.* 2008). The fortified rice kernels are blended with natural rice grains, typically in a 1:100 ratio. This technology has already proven beneficial in efficacy trials performed in Brazil, India, Mexico and other developing countries (Moretti *et al.* 2006; Hotz *et al.* 2008; Beinner *et al.* 2010).

In this experiment, we set out to create an extruded rice kernel to serve as a delivery vehicle for vitamin A, iron and zinc. Ascorbic acid was included to increase the availability of

iron (Hurrell *et al.* 2004). The extruded rice, which we named VitaRice, is a low cost, fortified rice that could be utilized to provide vital nutrients to a population where rice is a staple food. VitaRice is manufactured by an extrusion process that uses cheaper, broken rice to deliver a quick-cooking, fortified staple food. Unlike Ultra Rice, the fortified extruded rice kernels of VitaRice are not blended with natural grains but rather serve as the food itself. When preparing Ultra Rice, the super-fortified kernels must be thoroughly mixed into the natural grains prior to cooking. This raises the concern that if the mixing is not complete, some people may get a larger or smaller dose than intended. VitaRice does not have this issue because all of the grains carry equivalent amounts of the fortificants.

Fortification with iron can result in the development of off-flavors and unwanted color changes, so it was important to evaluate the sensory quality of the rice kernels using various iron compounds. Zinc oxide was chosen as a source of zinc, because it is an inexpensive fortificant that is commonly used in fortification programs around the world (Salgueiro *et al.* 2002). Zinc oxide does not interact with the food matrix and therefore does not cause sensory problems, but it is soluble in dilute acid, making it usable by the body (Salgueiro *et al.* 2002). Retinyl palmitate is a commonly used, highly bioavailable, pre-formed Vitamin A compound that is often used in supplementation and fortification programs (Carlier *et al.* 1993).

2.2 Objective

The objective of this project was to develop fortified, extruded rice kernels that would deliver the selected nutrients but maintain consumer acceptability. To achieve this

objective we fortified rice flour such that one serving of the final product would provide sufficient daily intake (based on published recommended dietary allowance values) of iron, zinc and vitamin A. We then analyzed for nutrient retention and color development in the fortified extruded rice.

2.3 Materials & Methods

Rice flour fomulations. Nine formulations were investigated in the course of this project. These formulations are presented in Table 2-1. For formulations T_2-T_5, the vitamins and minerals were obtained as premixes from Fortitech (Schenectady, NY, USA). For formulations, T_6-T_{10}, premixes were prepared in the lab using chemicals from Sigma-Aldrich (St. Louis, MO, USA). Micronized ferric pyrophosphate sourced from Dr. Paul Lohnmann Inc (Emerthal, Germany). With the exception of T_1 (Control), all treatments included iron added to furnish 18 mg per 100 gram flour.

For treatments containing zinc (as zinc oxide), vitamin A (as retinyl palmitate) and/or ascorbic acid, premixes were formulated to furnish 11 mg zinc, 4995 IU vitamin A and/or 70 mg ascorbic acid per 100 gram flour. For each treatment, long-grain white rice flour (Riviana; Houston, TX, USA) with 10% (wt/wt) moisture content was thoroughly combined with the appropriate premix and 0.5% (wt/wt) Dimodan®, a distilled monoglyceride emulsifier made from hydrogenated rapeseed oil (Dansico; Copenhagen, Denmark). Ingredients were mixed for 30 minutes in a wheelbarrow style mixer (Kushan Products, Inc.; Goldendale, WA, USA). Commercially available parboiled rice for comparison was

Table 2-1: Extruded rice formulations.

Treatment		Iron Compound	Other Mineral/Vitamin Fortificants
T_1	Control	*none*	*none*
T_2	FS-ZAC	Ferrous sulfate	Zinc oxide, retinyl palmitate, ascorbic acid
T_3	FS-ZC	Ferrous sulfate	Zinc oxide, ascorbic acid
T_4	EF-ZAC	Electrolytic iron	Zinc oxide, retinyl palmitate, ascorbic acid
T_5	EF-ZC	Electrolytic iron	Zinc oxide, ascorbic acid
T_6	mFPP-ZAC	Micronized ferric pyrophosphate	Zinc oxide, retinyl palmitate, ascorbic acid
T_7	mFPP-ZC	Micronized ferric pyrophosphate	Zinc oxide, ascorbic acid
T_8	mFPP	Micronized ferric pyrophosphate	*none*
T_9	EF	Electrolytic iron	*none*

purchased from P&C Supermarket (Ithaca, NY, USA). Parboiled rice was chosen because VitaRice is partially gelatinized and will cook more similarly to parboiled rice than non-parboiled rice grains.

Extrusion conditions. Extruded rice samples were prepared using a pilot-scale TX-52 Magnum co-rotating twin-screw extruded (Wenger Manufacturing; Sabetha, KS, USA) installed in Cornell University's Food Processing and Development Laboratory (Ithaca, NY, USA). For this project, the extruder was fitted with a barrel that had an L/D ratio of 15. The pressure in the extruder barrel was allowed to build up to a peak pressure of 500 psi using a customized screw configuration and a restrictor valve placed near the end of the extruder barrel. To cool the slurry and prevent puffing of the rice kernels as they emerged from the extruder barrel, cooled water was circulated around the barrel (35°C). The screw speed was set at 180 rpm, with the dry feed rate at 70 kg/hr. 10% (wt/wt) steam was injected into the preconditioner and 28% (wt/wt) water was added to the barrel to make the slurry. The temperature of the extruded dough was measured to be 85°C. The dough was shaped by extruding through a die support with rice kernel shaped inserts and cut by a rotating blade spinning at 1336 rpm. Extruded rice kernels were dried in a mechanical drier at 35°C to a moisture content of 10%.

Color Measurements. Color measurements were performed on the uncooked extruded rice kernels using a Macbeth ColorEye spectrophotometer (model #M2020PL) with the Opticview ProPalatte 5.0 software installed (Gretag Macbeth LLC; Grand Rapids, MI, USA). L-values correspond to lightness/darkness with higher values corresponding to more lightness. *a* and *b* values correspond to an object's color dimensions, with *a*-values describing

a sample's red to greenness, while *b*-values describe a sample's yellow to blueness. Larger *a* values indicate more redness and larger *b* values indicate more yellowness. The parameter, ΔE, defined as

$$\Delta E = [(L_1-L_2)^2 + (a_1-a_2)^2 + (b_1-b_2)^2]^{(1/2)}$$

was used to determine the overall color difference between each of the fortified samples and the unfortified control (Moretti *et al.* 2005).

Analysis of nutrient retention: Iron, Zinc, Vitamin A. To determine the vitamin and mineral content of the fortified flour, flour samples were collected from three different locations in the mixer immediately after mixing was complete. Samples of the dried, extruded rice were collected at the beginning of extrusion process, at the middle and at the end to ensure that all samples were thoroughly mixed. Due to time and cost constraints, only formuations T_1-T_5 were analyzed for mineral and vitamin A content.

For mineral analysis, rice flour and rice kernel samples were packaged in individual sealed containers and sent to the USDA Plant Soil and Nutrition Laboratory (Cornell University). There, samples were analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-AES). For vitamin A content analysis, fortified flour and extruded rice samples were packaged into individual, sealed containers and sent to Medallion Laboratory (Minneapolis, MN, USA) for analysis. There, they analyze for vitamin A following AOAC Official Method 2001.13. Briefly, samples are saponified in basic ethanol-water solution, neutralized, and diluted, converting retinol esters to retinol. Retinol content is quantified using HPLC (2000a).

Analysis of Ascorbic acid content. Ascorbic acid content was determined using AOAC Official Method 967.21 (2000b). In this method, ascorbic acid is extracted with a metaphosphoric acid-acetic acid solution and then titrated with 2,6-dicholoroindophenol, a oxidation-reduction indicator dye. In acidic solutions, 2,6-dichloroindophenol is red, but in the presence of ascorbic acid, it is reduced to a colorless species.

Solutions: (1) Metaphosphoric acid-acetic acid solution.—15 g HPO_3 pellets were dissolved in 40 mL glacial acetic acid and 200 mL ddH_2O; mixture was diluted to 500 mL and filtered rapidly through filter paper into a glass-stoppered bottle. Solution was stored at 4°C and a new solution was prepared weekly. *(2) Indophenol titrant solution.*—105 mg sodium bicarbonate ($NaHCO_3$) was added to 150 mL H_2O, and then 125 mg 2,6-dichloroindophenol Na salt was added and shaken until the dye dissolved. The solution was diluted to 500 mL and quickly filtered into an amber-glass stoppered bottle. Solution was stored at 4°C and a new solution was prepared weekly. Dye was standardized daily before use.

Titration procedure.—Rice samples were finely ground using a coffee grinder and then 4 grams were accurately weighed into a clean, 50 mL centrifuge tube. One control sample was spiked with 1 to 2 mg ascorbic acid to determine retention through the extraction process. 20 mL of the metaphosphoric acid-acetic acid solution was added to each tube. The tubes were vortexed vigorously and then allowed to rest for 5 minutes. The vortex-rest step was repeated two more times and then the samples were centrifuged at 8,000 x g. Four mL of the supernatant was removed and added to 5 mL of metaphosphoric acid-acetic acid solution. Solution was titrated to until persistant rose pink color remained for at least 5

seconds. Ascorbic acid concentration was determined based on the ml of dye used to titrate each sample compared to the standard curve.

Data analysis. Statistical analysis for micronutrient analysis and color measurement was performed using JMP 7.0 for Windows (SAS Institute; Cary, NC, USA). One-way ANOVA was used to determine if there was any significant difference. When significant difference was detected by ANOVA, Tukey-Kramer HSD test (which compares all pairs) was used to differentiate which samples were significantly different. Means were considered different at $p < 0.05$.

2.4 Results & Discussion

Color measurements. Table 1 displays the color profiles of the nine fortified extruded rice samples, the unfortified extruded rice control, and purchase commercially prepared parboiled rice. The two rice samples fortified with ferrous sulfate (FS-ZAC and FS-ZC) showed the largest color differences when compared to the control. FS-ZAC and FS-ZC had ΔE values of 17.36 and 17.80, respectively, with noticeable and unacceptable brown-gray discoloration of the rice kernels. These kernels exhibited decreased whiteness (*L* values) and increased blueness (*b* values). These findings are in line with previously published data, as ferrous sulfate reacts easily and quickly with the food matrix producing off colors (Moretti *et al.* 2005; Kongkachuichai *et al.* 2007).

Multinutrient fortification with micronized ferric pyrophosphate (mFPP-ZAC and mFPP-ZC) also resulted in grains that would be unacceptable to the consumer. These grains had a noticeable brown-discoloration that appeared more similar to natural brown rice grains

than white rice grains. This same effect was not observed in the rice sample containing only micronized ferric pyrophosphate (mFPP), which had a measurable, but not visually noticeable, decrease in whiteness (*L* values). It was expected that mFPP would appear similar to the unfortified control because micronized ferric pyrophosphate (0.5 μm) does not usually cause color changes in foods (Richins *et al.* 2008), and has been demonstrated to produce a color-acceptable extruded rice (Moretti *et al.* 2005; Beinner *et al.* 2010). It was not expected that samples mFPP-ZAC and mFPP-ZC would develop discoloration, however, it is possible that the color development is due to increased iron solubility due to the addition of ascorbic acid to the formulation.

The smallest color differences were observed for the rice samples containing only electrolytic iron (EF), containing electrolytic iron co-fortified with zinc and ascorbic acid (EF-ZC) and containing electrolytic co-fortified with zinc, retinyl palmitate and ascorbic acid with ΔE values of 0.90, 2.53 and 7.73, respectively. Multinutrient fortification with electrolytic iron (EF-ZAC) produeced a measurable discoloration of the kernels (decreased whiteness), but visually, the rice samples fortified with electrolytic iron appeared similar to the unfortified control. Only the rice sample fortified solely with electrolytic iron resulted in grains not significantly darker than the control. This is not in agreement with another published study on fortified, extruded grains. Moretti *et al.* (2005) found that electrolytic iron colored the extruded kernels a noticeable whitish-gray color, however, we did not see this discoloration, probably due to the level of fortificants used. Those authors prepared a rice similar to Ultra Rice that was designed to be mixed into natural grains at a ratio of 1:100 or

Table 2-2: Color profile of extruded rice samples and the commercial control. Means ± standard deviations (in parentheses). Values with different letters within the same column are significantly different, $p<0.05$.

Treatment	**L**	***A***	***b***	**ΔE***
Commercial parboiled rice	54.29 [a] (0.60)	4.74 [b] (0.18)	17.73 [a] (0.31)	3.03
T_1: Control	54.33 [a] (1.00)	3.78 [ef] (0.08)	14.87 [c] (0.13)	—
T_2: FS-ZAC	37.59 [g] (0.43)	3.73 [f] (0.16)	10.24 [h] (0.20)	17.36
T_3: FS-ZC	37.07 [g] (0.70)	4.16 [c] (0.09)	10.51 [h] (0.24)	17.80
T_4: EF-ZAC	46.80 [d] (0.77)	3.41 [g] (0.10)	13.63 [d] (0.18)	7.73
T_5: EF-ZC	51.84 [b] (0.75)	3.75 [f] (0.05)	15.36 [b] (0.10)	2.53
T_6: mFPP-ZAC	43.69 [e] (0.83)	5.05 [a] (0.07)	12.34 [e] (0.20)	11.01
T_7: mFPP-ZC	39.23 [f] (0.25)	4.87 [ab] (0.04)	11.47 [f] (0.03)	15.52
T_8: mFPP	50.08 [c] (0.96)	3.94 [de] (0.10)	13.84 [d] (0.17)	4.37
T_9: EF	55.04 [a] (0.14)	4.04 [cd] (0.04)	15.36 [b] (0.08)	0.90

*The ΔE parameter compares the *Lab* values for each sample to the *Lab* values of the extruded, unfortified control (T_1).

1:200. As such, their levels of iron fortification were 0.5 or 1 g/100 g rice, considerably higher than our level of 18 mg/100 g rice.

At our level of use, electrolytic iron produced the least amount of discoloration an produced an extruded rice with a visual appearance most similar to the unfortified, extruded control rice.

Nutrient Retention. Iron, zinc, vitamin A and ascorbic acid concentrations are presented in Table 2-3.

Iron & Zinc. For iron, it was noted that in all cases, the measured values were higher than the intended target, but all values were in the range of 18-21 mg iron/100 g flour. For zinc, those samples containing zinc (EF-ZAC and EF-ZC) had measured values higher than the intended target, but within 12-14 mg zinc/100 g flour. Excellent retention of both iron and zinc were observed, with the % mineral retention between 94-112%. These results are very high, but were expected as iron and zinc are not heat or light sensitive, and it would not be expected that significant losses would occur during processing (Moretti *et al.* 2005).

Vitamin A Retention. A significant decrease in vitamin A occurred during extrusion. Vitamin A retention in extruded rice treatments FS-ZAC and EF-ZAC was only 58% and 52%, respectively, compared to the corresponding fortified flour. The loss of vitamin A from fortified flour to extruded rice samples was likely due to heat destruction during the extrusion and drying process. The destruction of 42-48% of the retinyl palmitate is high but within published values (3-48% destruction) (Björck & Asp 1983).

Vitamin C Retention. Table 2-3 displays the measured ascorbic acid concentrations for six of the fortified rice samples plus the control. Ascorbic acid analysis was not

performed on the samples fortified with ferrous sulfate due to known interferences of the ferrous ion with the analysis method used. It was also determined that the darkened color of the ferrous sulfate-fortified kernels would not be acceptable to consumers, and did not have potential for use.

The ascorbic acid content of the fortified flours was measured to verify the original goal of 70 mg ascorbic acid per 100 g flour. The measured ascorbic acid concentrations of the flour samples fortified with electrolytic iron and soluble ferric pyrophosphate achieved this target. However, the flours fortified with micronized ferric pyrophosphate (mFPP-ZAC and mFPP-ZC) demonstrated slightly lower initial ascorbic acid contents. The ascorbic acid concentrations of all extruded rice samples showed a decrease in ascorbic acid when compared to the fortified flour. The samples fortified with electrolytic iron (EF-ZAC and

Table 2-3: Nutrient analysis of fortified extruded rice samples.

Nutrient	Treatment	Rice Flour	Extruded Rice	Nutrient retention (after extrusion)
Iron (mg/100 g)	T_1: Control	<5	<5	ND
	T_4: EF-ZAC	21.44 [a] (0.43)	21.72 [a] (0.25)	101%
	T_5: EF-ZC	21.69 [a] (0.19)	21.12 [a] (0.51)	97%
	T_8: mFPP	21.40 [a] (0.14)	20.07 [b] (0.06)	94%
	T_9: EF	18.19 [a] (0.21)	20.45 [b] (0.03)	112%
Zinc (mg/100 g)	T_1: Control	ND	ND	ND
	T_4: EF-ZAC	13.60 [a] (2.57)	12.93 [b] (0.19)	95%
	T_5: EF-ZC	12.72 [a] (0.17)	12.79 [a] (0.10)	100%
Retinyl palmitate (IU/100 g)	T_1: Control	<50	ND	ND
	T_2: FS-ZAC	4390 [a] (130)	2535 [b] (280)	58%
	T_3: FS-ZC	ND	ND	ND
	T_4: EF-ZAC	4785 [a] (130)	2485 [b] (200)	52%
	T_5: EF-ZC	ND	ND	ND
Ascorbic acid (mg/100 g)	T_1: Control	<2.5	<2.5	ND
	T_4: EF-ZAC	70.2[a] (4.6)	56.7 [b] (2.6)	81%
	T_5: EF-ZC	69.7 [a] (2.5)	51.9 [b] (0.8)	74%
	T_6: mFPP-ZAC	60.2 [a] (5.10)	45.7 [b] (1.4)	76%
	T_7: mFPP-ZC	59.9 [a] (7.10)	33.6 [b] (1.0)	56%
	T_8: mFPP	<2.5	<2.5	ND
	T_9: EF	<2.5	<2.5	ND

Means with different letters within a row are significantly different (p <0.05). Figures in parenthesis represent standard deviation. ND=not determined, these samples were not fortified with the indicated nutrient.

EF-ZC) showed the highest ascorbic acid retention values, 81% and 74% respectively. The sample fortified with micronized ferric pyrophosphate and vitamin A (mFPP-ZAC) showed a moderate retention value of 76% ascorbic acid. The lowest ascorbic acid retention values were for the rice sample fortified with micronized ferric pyrophosphate (mFPP-ZC) with values of 56%, respectively.

It was noted that the darker samples (mFPP-ZAC and mFPP-ZC) had lower ascorbic acid retention than the lighter samples (EF-ZAC and EF-ZC). This suggested the possibility that the developed color was due to the oxidation of ascorbic acid, however, this hypothesis was not tested during the course of this experiment.

These results indicate that it is feasible to create fortified, extruded rice kernels that would be acceptable for consumers. As mentioned previously, the Ultra Rice project is making great strides to correct nutrient deficiencies around the world, but the advantage of the fortified rice investigated in this project (VitaRice) is that it is easier to use. Every kernel of VitaRice contains the selected nutrients and one can be assured that every serving would have the same amount of each nutrient. An important step for future research is to conduct cooking trials to study the potential loss of Vitamin A and ascorbic acid in cooking. As well, it would be crucial to conduct long-term shelf life studies in storage conditions similar to those in the target geographical areas.

2.5 Conclusions

The objective of this project was to create fortified, extruded rice that would deliver selected nutrients without undesirable color changes. The results presented here demonstrate that with respect to color, if the grains will be co-fortified with zinc, vitamin A and ascorbic acid, electrolytic iron is the best choice for fortified, extruded rice kernels. The other treatments, which utilized ferrous sulfate or micronized ferric pyrophosphate, produced noticeably discolored rice kernels. Excellent retention of iron and zinc after extrusion was observed (94-112%). Significant processing loss was observed for vitamin A (42-48% destruction) and ascorbic acid (19-44% destruction), although retention in the electrolytic iron-fortified samples was highest (74-81% ascorbic acid retention). Therefore, extruded rice fortified with iron (as electrolytic iron), zinc, vitamin A and ascorbic acid could be an effective vehicle to address the issue of micronutrient malnutrition among a rice eating populace.

CHAPTER 3: EFFECT OF CO-FORTIFICATION ON IRON DIALYZABILITY

EFFECT OF VITAMIN A AND ASCORBIC ACID ON IRON DIALYZABILITY

3.1 Rationale

The previous chapter described the development of extruded rice, fortified with iron, zinc, vitamin A and/or ascorbic acid. The fortified kernels were envisioned for use in rice eating countries as a strategy for correcting micronutrient deficiencies. The fortified extruded rice demonstrated excellent retention of minerals and acceptable retention of vitamins after processing. The electrolytic iron-fortified samples demonstrated the least amount of color development, while the ferrous sulfate-fortified samples darkened to an unacceptable level. It was decided that the rice sample EF-ZAC, which contained electrolytic iron (21 mg/100 g rice), zinc oxide (13 mg/100 g rice), retinyl palmitate (2,000 IU/100 g rice) and ascorbic acid (55 mg/100 g rice), had the most potential for use. However, the previous study did not investigate the bioavailability of this rice. The present investigation uses *in vitro* digestion to approximate the bioavailability of the extruded rice samples in humans (Miller *et al.* 1981). *In vitro* digestion has proved to be an effective tool for surveying and estimating bioavailability of many food samples without incurring the large cost and time expense of human trials (Fairweather-Tait *et al.* 2005; Argyri *et al.* 2011). It is certainly not a substitute for performing absorption studies in humans, but it is an excellent survey technique (Argyri *et al.* 2011).

The i*n vitro* digestion method simulates the conditions of digestion in the gastrointestinal tract and then monitors the amount of dialyzable ferrous iron produced.

This was achieved by lowering the sample pH to 2 and adding the digestive enzyme pepsin for 2 hour incubation at human body temperature (37°C). Then the pH was gradually raised by dialyzing PIPES buffer into the digest, and bile salts and pancreatin were added for additional 2 hour incubation at 37°C. This dialysis method allowed for the differentiation of soluble, low molecular weight iron complexes from soluble high molecular weight iron complexes.

3.2 Approach

The iron fortified extruded rice was cooked in a rice cooker and digested using a modified procedure based on a previously described method (Miller *et al.* 1981; Kapsokefalou & Miller 1991). The extruded rice samples chosen were treatments EF-ZAC (electrolytic iron with zinc, vitamin A and ascorbic acid), EF-ZC (electrolytic iron with zinc and ascorbic acid), EF (electrolytic iron only), mFPP-ZAC (micronized ferric pyrophosphate with zinc, vitamin A and ascorbic acid), mFPP-ZC (micronized ferric pyrophosphate with zinc and ascorbic acid), mFPP (micronized ferric pyrophosphate only). All samples, except mFPP-ZAC and mFPP-ZC, were deemed to be acceptable to consumers because the color and shape of the extruded grains were similar to those of the commercially purchased parboiled rice grains. Rice treatments mFPP-ZAC and mFPP-ZC produced discolored gains that were judged to be unacceptable to consumers. However, these grains were included in the *in vitro* digestion to determine if the presence of vitamin A and/or ascorbic acid would increase the amount of dialyzable iron, as compared to rice treatment mFPP.

3.3 Materials

Distilled, deionized water was used throughout the experiment. All glassware was washed with detergent, rinsed with water, soaked overnight in 1 M HCl, rinsed and dried before use. All chemicals used were sourced from Sigma Aldrich (St. Louis, MO, USA), unless otherwise noted.

Ascorbic acid determination solutions. Refer to Section 2.3 for preparation instructions. Metaphosphoric acid-acetic acid solution (15 g HPO_3 pellets dissolved in 40 mL glacial acetic acid and diluted to 500 mL) and indophenol titrant solution (125 mg 2,6-dichloroindophenol Na salt added to 150 mL ddH_2O containing 105 mg sodium bicarbonate ($NaHCO_3$).

Nonheme ferric iron. Iron Atomic Absorption Standard Solution (1000 μg/mL in 1% wt HCl) was used as the source of Fe (III) in all treatments.

Pepsin. 4.0 g porcine pepsin preparation was suspended in 0.1 M HCl, and diluted with 0.1 M HCl to 100 mL.

Pancreatin/bile mixture. 0.2 g porcine pancreatin and 1.2 g bile extract was suspended in 0.1 M $NaHCO_3$ and diluted to make 100 mL

PIPES buffer, 0.15 M, pH 6.1. PIPES (piperazine-N,N'-bis[2-ethane-sulfonic acid] disodium salt) was dissolved in water at a concentration of 0.15 M, and adjusted to pH 6.1 with concentrated HCl.

HEPES buffer, 0.3 M, pH 9.9. HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid sodium salt) in water at a concentration of 0.3 M, no pH adjustment.

Reducing protein precipitant solution. 100 g trichloroacetic acid and 50 g hydroxylamine monohydrochloride were dissolved in 300 mL water. 100 mL concentrated HCl was added and the solution was brought to 1L with water.

Non-reducing protein precipitant solution. 100 g trichloroacetic acid was dissolved in 300 mL water and 100 mL concentrated HCl was added. Solution was bought to 1L with water

BPDS solution, 5.3 mg/mL. Bathophenanthroline disulfonic acid solution in water.

Dialysis tubing. Spectra/Por Dialysis Membrane 7 tubing with a molecular weight cut-off of 8000 (Spectrum Laboratories; Rancho Dominguez, CA, USA) was cut into 15 cm lengths and soaked in water for 1 hour before use.

3.4 Methods

Sample preparation. Rice grains were added at a water to rice ratio of 1:1 (v/v) to an electric rice cooker (Panasonic; Secaucus, NJ, USA). Cooking time was determined automatically by the rice cooker based on a temperature sensor (approximately 12-15 minutes). After the cooking, rice was allowed to sit for 5 minutes in the rice cooker and 25 minutes on the bench top prior to use for ascorbic acid content analysis and *in vitro* digestion.

Ascorbic acid determination. 2 grams of cooked rice from each treatment, in triplicate, were weighed into 50 mL screw-cap centrifuge tubes. 10 mL of metaphosphoric acid-acetic acid solution was added and the rice kernels were pulverized with a glass rod. The slurries were vortex mixed and allowed to sit for 5 minutes, and then the vortex mix/rest process

was repeated 2 more times. The slurries were centrifuged at 8,000 x g for 10 minutes and then 4 mL of the supernatant was removed and titrated with the indophenol solution until a rose pink color persisted at least 5 seconds.

In vitro digestion. A modification of the in vitro digestion methods proposed by Kapsokefalou and Miller (1991) and (Miller *et al.* 1981) was used to assess iron dialyzability. 6 grams of cooked rice from each treatment, in triplicate, were weighed into 50 mL screw-cap centrifuge tubes and homogenized with 10 mL ddH_2O for 45 seconds using the bench top homogenizer, Polytron® CH 6010 (Kinematica; Lucerne, Switzerland). The pH of the slurries was adjusted to 1.7 with 6 M HCl and 1 mL of pepsin suspension was added. The samples were tightly capped and incubated in a shaking water bath at 37°C for 2 hours. Lengths of dialysis tubing were sealed at one end using Spectra/Por Closures (Spectrum Laboratories) and were filled with 20 mL PIPES buffer. After the 2 hour pepsin incubation, one dialysis bag was added to each tube and the samples were incubated at 37°C. After 30 minutes, 5 mL of the pancreatin-bile mixture was added to each tube and the tubes were incubated for an additional 2 hours. The dialysis bags were removed and rinsed by dipping in ddH_2O. The bag contents were transferred into 50 mL screw-cap centrifuge tubes. The dialysate and retentate tubes were centrifuged at 8,000 x g for 10 minutes.

Iron assay. Dialyzable iron concentrations were measured using a modification of the method proposed by (Reddy *et al.* 1986). For total iron determination, 2 mL of the reducing protein precipitant solution was added to a 4 ml aliquot of each dialysate. For ferrous iron determination, 2 mL of the non-reducing protein precipitant was added to each dialysate. Samples sat overnight at room temperature and then centrifuged at 12,000 x g for 10

minutes. 1.5 mL aliquots of the supernatants (in duplicate) were transferred to separate cuvettes and 0.35 mL BPDS solution was added, along with 1.0 mL HEPES buffer. Color was allowed to develop for 1 hour and then the absorbance was measured at 535 nm using a DU® 520 spectrophotometer (Beckman Coulter; Indianapolis, IN, USA). The blank was prepared by adding 1.5 mL 0.01 M HCl to a cuvette along with 0.35 mL BPDS and 1 mL HEPES buffer. Standards were prepared by adding various amounts of the iron atomic absorption standard solution to cuvettes along with 0.35 mL BPDS and 1.0 mL HEPES buffer. Color was allowed to develop for 1 hour before measuring the absorbance at 535 nm.

Calculations. Dialyzable ferrous iron (D-Fe(II)) and dialyzable total iron (D-(Fe(II) + (Fe(III))) were expressed as percentages of the total added iron in each tube. Similar to Kapsokefalou and Miller (1991), I assumed that dialyzable iron had equilibrated across the dialysis membrane during the 2.5 hour incubation time. The following equations were used to calculate dialyzable ferrous and total iron:

D-Fe(II):

$$\frac{^{1}[\mathrm{Fe(II)}]_{\mathrm{D}}(\mu g/mL) \times {}^{2}\text{total volume (mL)}}{\text{Fe (III) in orginal sample } (\mu g)} \times 100$$

[1]ferrous iron concentration in the dialysate
[2]dialysate volume + retentate volume

D-(Fe(II) + Fe(III)):

$$\frac{^{3}[\mathrm{Fe(II) + Fe(III)}]_{\mathrm{D}}(\mu g/mL) \times {}^{2}\text{total volume (mL)}}{\text{Fe (III)in orginal sample } (\mu g)} \times 100$$

[2]dialysate volume + retentate volume

[3]total iron concentration in the dialysate

Data analysis. Statistical analysis for ascorbic acid analysis and dialyzable iron was performed using JMP 7.0 for Windows (SAS Institute; Carey, NC, USA).

3.5 Results

Ascorbic acid retention. Table 3-1 shows the ascorbic acid contained in the extruded rice after cooking in an electric rice cooker. A conversion factor was needed in order to calculate ascorbic acid retention after extrusion and cooking, by comparing the amount of ascorbic acid in cooked rice to the amount in the unfortified flour. Cooked parboiled rice has a reported final moisture content of 60-65% (Saleh & Meullenet 2007; Ali *et al.* 2010). Assuming the moisture content is 60%, 100 grams of uncooked rice (moisture content 10%) will absorb sufficient water to make 225 grams of cooked rice (moisture content 60%). Therefore, the following conversion was used to convert the amount of ascorbic acid in cooked rice to uncooked rice:

$$(\chi \text{ mg ascorbic acid}/100 \text{ g cooked rice}) \times (225 \text{ g cooked rice}/100 \text{ g uncooked rice})$$

Retention values were compared to the pre-extrusion, fortified flour. Cooking resulted in a destruction of about half of the ascorbic acid present after extrusion but before cooking, which is in accordance with published results (Teucher *et al.* 2004).

Dialyzable iron. Figure 3-1 displays the dialyzable ferrous and total iron content measured from the cooked extruded rice after *in vitro* digestion. Table 3-2 presents the average and standard

Table 3-1: Effect of cooking on ascorbic acid content in the extruded rice samples. Means ± standard deviations (in parentheses) where indicated by "±".

	Ascorbic Acid Content		Nutrient Losses	
Treatment	**After Cooking (mg/100 g cooked rice)**	**After Cooking (mg/100 g uncooked rice equivalent)**	**Losses due to Cooking***	**Losses due to Extrusion & Cooking****
EF-ZAC	10.35 ± 0.41	23.3 ± 0.9	59%	67%
EF-ZC	13.70 ± 0.19	30.8 ± 0.4	41%	56%
mFPP-ZAC	7.50 ± 0.16	16.9 ± 0.4	63%	72%
mFPP-ZC	7.13 ± 2.11	16.0 ± 4.7	52%	73%

*Content values were compared to the extruded, uncooked rice kernels.
**Content values were compared to the pre-extrusion, fortified flour.

deviations for measured dialyzable ferrous and total iron. There were statistically significant differences between samples fortified with iron in the presence of zinc, ascorbic acid and vitamin A versus samples fortified with iron alone. EF-ZAC and EF-ZC were not statistically different from each other but were significantly different from EF. As well mFPP-ZAC and mFPP-ZC were not statistically different from each other but were statistically different from mFPP.

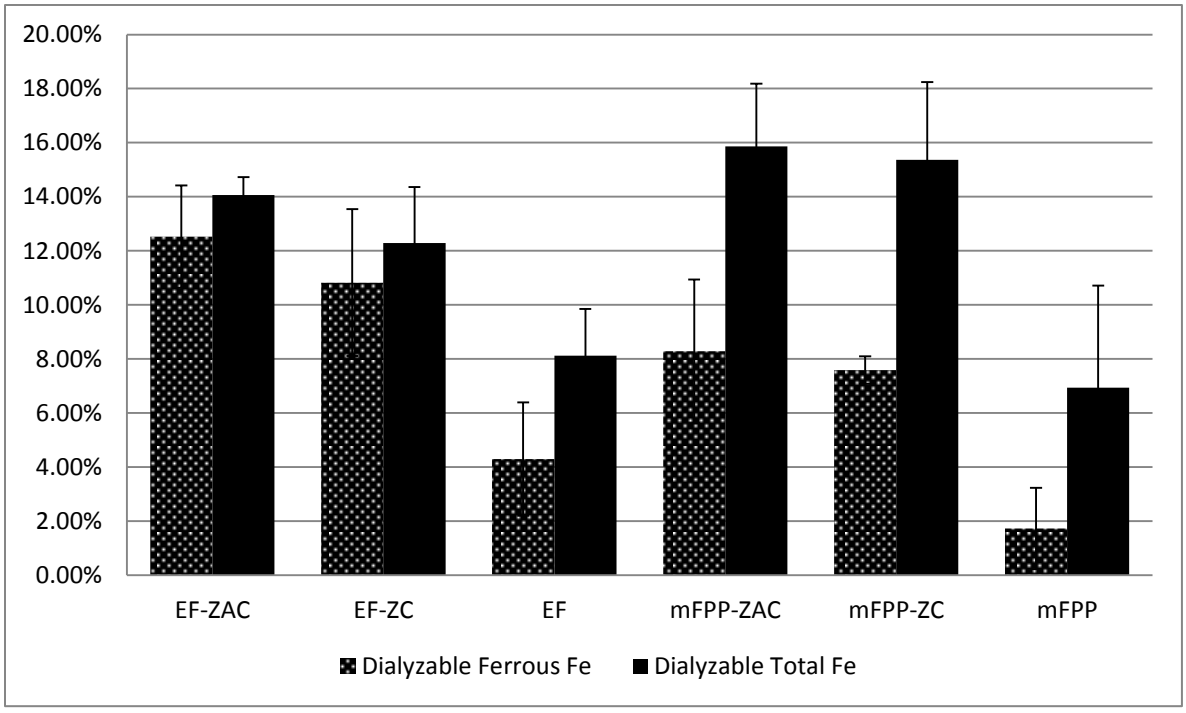


Figure 3-1: Dialyzable total and ferrous iron formed during *in vitro* digestion of extruded rice. Each value is mean ± standard deviation for three observations. See Table 3-2 for the results of the statistical analysis.

Table 3-2: Dialyzable total and ferrous iron formed during *in vitro* digestion of extruded rice. Means and standard deviations (in parentheses) for each rice treatment. Values in the same column and with the same iron fortificant used (EF or mFPP) with different letters are significant, $p<0.05$.

Treatment	Dialyzable Fe(II) (in %)	Dialyzable Total Fe (in %)
EF-ZAC	12.52 ^{A} (1.90)	14.07 ^{A} (0.66)
EF-ZC	10.82 ^{A} (2.72)	12.29 ^{AB} (2.07)
EF	4.30 ^{B} (2.10)	8.12 ^{B} (1.73)
mFPP-ZAC	8.28 ^{A} (2.66)	15.86 ^{A} (2.32)
mFPP-ZC	7.59 ^{A} (0.51)	15.36 ^{A} (2.87)
mFPP	1.72 ^{B} (1.50)	6.94 ^{B} (3.77)

3.6 Discussion.

The *in vitro* digestion method was initially proposed by (Miller *et al.* 1981) as a way to predict iron absorption. The methodology simulates the digestive process using pH changes, gastric enzymes and bile salts, which allows iron to fully interact with food components, similarly to within the gastrointestinal tract. The use of a dialysis bag allows for the gradual increase of pH similar to in the intestines but also permits small, soluble iron complexes to diffuse into the bag. Molecular weight is a factor that influences iron absorption (Miret *et al.* 2003), and the dialysis bag presumably separates the iron complexes that would be able to diffuse through the mucous layer to reach the brush border membrane of the enterocytes from insoluble and large molecular weight soluble iron complexes that are not able to reach the brush border. Therefore, it was proposed that the measurement of total dialyzable iron can predict iron bioavailability from a food. It was later proposed that dialyzable ferrous iron is a better indicator than total dialyzable iron for the prediction of iron bioavailability. Kapsokefalou and Miller (1991) observed that, for a series of samples, dialyzable ferrous iron corresponded better to published iron absorption data than dialyzable total iron. As such, this present investigation will use ferrous dialyzable iron as the basis for comparison of samples.

Iron dialyzability was increased in the treatments containing ascorbic acid compared to their respective iron-only treatment. These results support previous findings that the addition of ascorbic acid enhances dialyzability (Hallberg *et al.* 1986; Siegenberg *et al.* 1991). Ascorbic acid is thought to enhance iron dialyzability (*in vitro*) and absorption (*in vivo*) by reducing ferric iron to ferrous iron in the stomach, and then chelating the iron (Hurrell *et al.*

2004). This chelation prevents insoluble ferric hydroxide formation as the pH rises in the duodenum. Because of these unique reduction and chelation properties, ascorbic acid can overcome the inhibitory effects of phytate, polyphenols and other iron absorption inhibitors.

The fact that neither EF-ZAC and EF-ZC nor mFPP-ZAC and mFPP-ZC were significantly different implies that either vitamin A has no effect on iron dialyzability or that there is a plateau effect. It has been reported that the addition of ascorbic acid has a linear dose response which eventually levels off to a plateau (Hurrell *et al.* 2004). As such, it is possible that in our samples any effect of vitamin A addition was hidden by the effects of ascorbic acid. However, the plateau effect of ascorbic acid has been reported to occur close to a 7.5 molar ratio of ascorbic acid to iron, whereas all of our samples fell well beneath that ratio (~0.3-0.5 molar ratio ascorbic acid to iron). Therefore it was not likely that there was a plateau effect, but since vitamin A has been reported to increase iron absorption, further study was warranted (García-Casal 2006).

3.7 Conclusions

The previously developed EF-ZAC rice, fortified with electrolytic iron, zinc, retinyl palmitate and ascorbic acid, has the best potential for use in rice eating populations as a means for alleviating micronutrient deficiency. The addition of ascorbic acid to both iron-only fortified rice formulas (EF and mFPP) resulted in a 3-4 fold increase in iron dialyzability, but since the multinutrient formulas containing micronized ferric pyrophosphate (mFPP-ZAC and mFPP-ZC) were deemed unacceptably discolored, only EF-ZAC and EF-ZC have potential for use. It is not known if the addition of vitamin A

would increase iron absorption from the cooked rice, but since it does not decrease the effects of ascorbic acid, EF-ZAC rice has the most potential for use.

EFFECT OF VITAMIN A ON IRON DIALYZABILITY

3.8 Rationale

Vitamin A is essential for proper iron metabolism and individuals with vitamin A deficiencies will show depressed hemoglobin concentrations, even if their diet contains adequate iron content (Hodges *et al.* 1978). It has been suggested that vitamin A deficiency disrupts erythropoietin synthesis (Davidsson *et al.* 2003), reduces uptake of iron by the bone marrow (Beynen *et al.* 1992; Sijtsma *et al.* 1993), impairs differentiation of blood cells (Schroeder *et al.* 1992), and reduces mobilization of iron from body stores (Mejia & Arroyave 1982). As well, populations with a high prevalence of vitamin A deficiency have increased risk of infection, and the presence of infection causes the body to sequester iron, thereby decreasing hemoglobin concentrations (Semba & Bloem 2002). A positive correlation between serum retinol content and hemoglobin concentration has been reported in several cross-sectional studies (Walczyk *et al.* 2003).

Authors from Venezuela reported that the addition of vitamin A or β-carotene to an iron-fortified meal increased the amount of iron absorbed from the meal (Layrisse & Garcia-Casal 1997; Garcia-Casal *et al.* 1998). However, these findings have been contradicted by another research group's findings that vitamin A did not increase iron absorption (Davidsson *et al.* 2003; Walczyk *et al.* 2003). The research group that reported the positive enhancement used a method of cooking the test foods with added retinyl palmitate or

β-carotene (Garcia-Casal *et al.* 1998), while the other research group added the retinyl palmitate immediately prior to consumption (Walczyk *et al.* 2003). This present study investigates if the point of retinyl palmitate addition contributed to the outcomes reported.

3.9 Objective

The objective of this experiment was to determine if there existed any differences in iron dialyzability between the control corn porridge (containing only ferrous sulfate) and the samples fortified with either vitamin A or β-carotene. Additionally, the secondary objective was to determine if a different outcome would be achieved if the vitamin A/β-carotene was added prior to cooking or immediately before digestion.

3.10 Materials

Distilled, deionized water was used throughout the experiment. All glassware was washed with detergent, rinsed with water, soaked overnight in 1 M HCl, rinsed and dried before use. All chemicals used were sourced from Sigma Aldrich (St. Louis, MO, USA), unless otherwise noted.

Corn porridge. Corn flour ("polenta") was purchased from the local grocer GreenStar (Ithaca, NY, USA). Sucrose and corn oil were purchased from the local grocer ALDI (Ithaca, NY, USA). Water dispersible β-carotene 10% was sourced from Hoffmann-La Roche (Nutley, NJ, USA)

In vitro digestion solutions. Refer to Section 3.3 for preparation instructions. Pepsin suspension (4.0 g porcine pepsin preparation in 0.1 M HCl to make 100 mL), pancreatin/bile

mixture (0.2 g porcine pancreatin and 1.2 g bile extract in 0.1 M $NaHCO_3$ to make 100 mL), PIPES buffer (0.15 M PIPES, adjusted with pH 6.3 with concentrated HCl), HEPES buffer (0.3 M HEPES, no pH adjustment), reducing protein precipitant solution (100 g trichloroacetic acid, 50 g hydroxylamine monohydrochloride and 100 mL concentrated HCl per 1 L of water), non-reducing protein precipitant solution (100 g trichloroacetic acid and 100 mL concentrated HCl per 1 L of water), bathophenanthrolinedisulfonic solution (BPDS, 5.3 mg/mL), and Spectra/Por Dialysis Membrane 7 tubing.

3.11 Methods

Treatments. There was one control corn porridge, fortified only with ferrous sulfate. Retinyl palmitate was added to two treatments (either before or after cooking). β-carotene was added to two treatments (either before or after cooking). This information is presented in Table 3-3.

Table 3-3: Description of corn porridge treatments.

Treatment	Sample Description
T1	Retinyl palmitate added prior to cooking
T2	Retinyl palmitate added after cooking
T3	Control
T4	β-carotene added prior to cooking
T5	β-carotene added after cooking

Sample preparation. Stiff corn porridges were prepared from 3 g of corn flour ("polenta"), 12 g water, 0.4 g sugar, and 0.3 g corn oil, according to a modified procedure based on the method used by Davidsson *et al* (2003). The corn flour, water, sugar and oil were added to a 50 mL centrifuge tube along with 1.2 mg of Fe, as $FeSO_4$. For samples T1 and T4, retinyl palmitate and β-carotene, respectively, were added to the tubes as well. Both were added such that the addition was equivalent to 65 μg RE (65μg retinyl palmitate added and 390 μg β-carotene added). Tubes were swirled to mix. The tubes were placed into boiling water bath and cooked until most of the water was absorbed or evaporated. Samples were immediately diluted to 25.0 mL using room temperature distilled water, capped, inverted several times to mix and allowed to cool on their sides. Once samples were cooled, retinyl palmitate and β-carotene were added to samples T2 and T5, respectively and inverted several times to mix.

***In vitro* digestion.** The pH was adjusted to 1.7 using 1:1 diluted concentrated HCl and 1 mL of pepsin suspention was added. The samples were tightly capped and incubated in a shaking water bath at 37°C for 2 hours. Dialysis bags were sealed at one end using Spectra/Por plastic clamps and then filled with 15 mL PIPES buffer. One filled dialysis bag was added to each tube and tubes were added back to the shaking water bath. After 30 minutes, 5 mL of the pancreatin-bile mixture was added to each tube and the tubes were incubated for an additional 2 hours. The dialysis bags were removed from the tube and rinsed by dipping in ddH_2O. The contents of the dialysis bags were emptied into clean 50 mL centrifuge tubes.

Iron assay. The dialysate and retentate tubes were centrifuged at 12,000 x g for 10 minutes. For total iron determination, 2 mL reducing protein precipitant solution was added to a 4 mL aliquot of each dialysate supernatant. For ferrous iron determination, 2 mL non-reducing protein precipitant solution was added a 4 mL aliquot of each dialysate supernatant. The samples were allowed to sit overnight at room temperature. The samples were centrifuged at 12,000 x g for 10 minutes and then 1.5 mL aliquots of the supernatants (in duplicate) were transferred to separate cuvettes. 0.35 mL BPDS solution was added and along with 1 mL HEPES buffer. Samples were allowed to sit at room temperature for 2 hours to allow color to develop. Absorbance was measured at 535 nm using a spectrophotometer (DU® 520, Bechman Coulter).

Calculations. Refer to Section 3.4 for the equations to calculate dialyzable ferrous iron and dialyzable total iron.

Data analysis. Statistical analysis for ascorbic acid analysis and dialyzable iron was performed using JMP 7.0 for Windows (SAS Institute; Carey, NC, USA).

3.12 Results

Dialyzable iron. Figure 3-2 displays the dialyzable ferrous and total iron content measured from the cooked extruded rice after *in vitro* digestion. Table 3-4 presents the average and standard deviations for measured dialyzable ferrous and total iron. Neither retinyl palmitate nor β-carotene samples were significantly different from the control. The addition of retinyl palmitate or β-carotene prior to cooking appeared to decrease iron

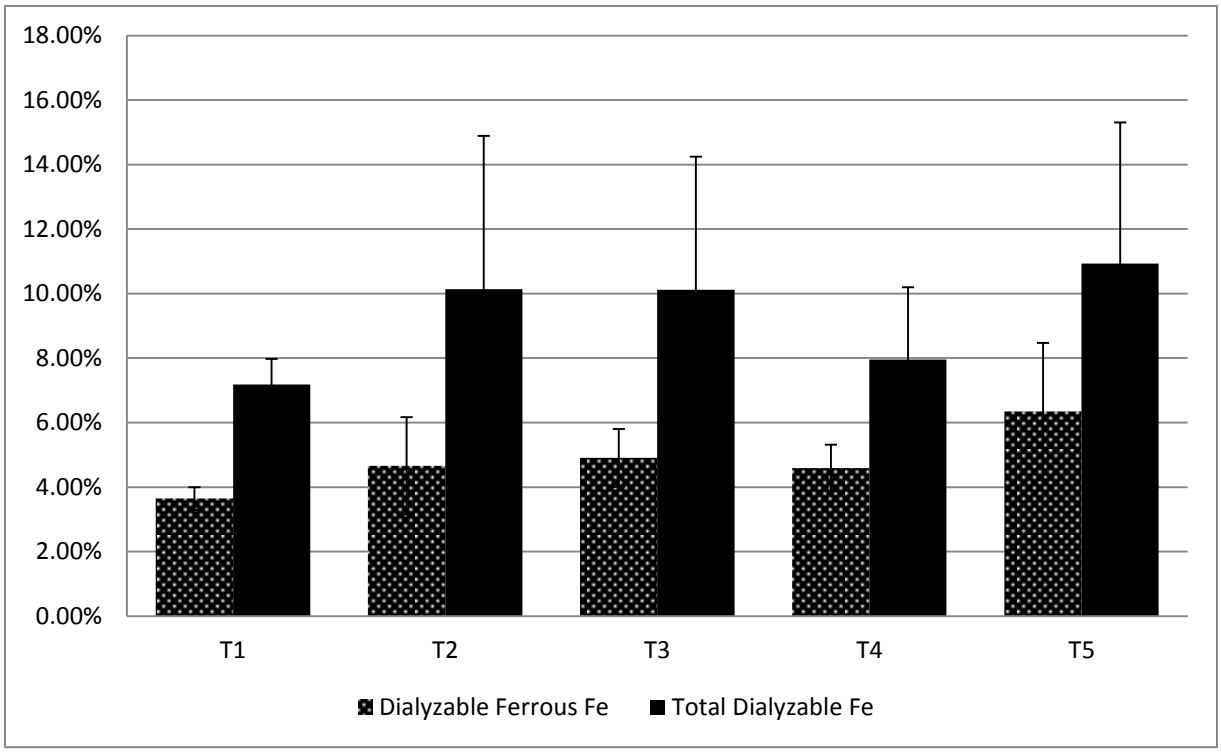


Figure 3-2: Dialyzable total and ferrous iron formed during *in vitro* digestion of corn porridges. Each value is mean ± standard deviation for three observations. Treatments: T1 (retinyl palmitate precook), T2 (retinyl palmitate postcook), T3 (control), T4 (β-carotene precook) and T5 (β-carotene postcook). See Table 3-4 for results of statistical analysis.

Table 3-4: Dialyzable total and ferrous iron formed during *in vitro* digestion of corn porridges. Means and standard deviations (in parentheses) for each corn porridge treatment. Values in the same column with different letters are significant, $p<0.05$. Values in a column without letters indicates that all values in that column are not statistically different. Treatments: T1 (retinyl palmitate precook), T2 (retinyl palmitate postcook), T3 (control), T4 (β-carotene precook) and T5 (β-carotene postcook).

Treatment	Dialyzable Fe(II) (in %)	Dialyzable Total Fe (in %)
T1	3.64^{B} (0.34)	7.18 (0.80)
T2	5.14^{BA} (1.69)	10.14 (4.76)
T3 (Control)	5.19^{BA} (1.00)	10.12 (4.13)
T4	4.81^{BA} (0.80)	7.95 (2.24)
T5	7.03^{A} (2.37)	10.93 (4.37)

dialyzability slightly as compared to the addition of retinyl palmitate or β-carotene after cooking, but this observation was not statistically significant.

3.13 Discussion

It has been reported that the addition of vitamin A (as retinyl palmitate) to a ferrous fumarate-fortified corn bread significantly increased iron absorption from a meal, even in the presence of the known inhibitor, coffee (Layrisse & Garcia-Casal 1997; Garcia-Casal *et al.* 1998). The authors suggested that vitamin A acts as a chelating agent, complexing the iron during the digestive process, thereby preventing the inhibitory effect of phytate and polyphenols on nonheme iron absorption. However, this position was challenged by the observation that the molar ratios of vitamin A to iron in the Venezuelan corn bread were very low after baking and storage (≈0.004:1-0.008:1), and therefore solely the formation of these complexes does not explain the 2 to 4 fold increase in absorption that Layrisse, Garcia-Casal and colleagues reported. A follow up study performed in Switzerland by Walczyk *et al.* (2003) did not find that vitamin A increased iron absorption from a ferrous fumarate-fortified cornbread. It was suggested that the socioeconomic status of the participants in the study could explain the differences in observed effects. In the Venezuelan studies, adult subjects were selected from a low socioeconomic stratum and some of the women had moderate iron deficiency anemia, whereas in the Swiss study, healthy university students and staff from presumably higher socioeconomic strata were used in the experiment. If the Venezuelan subjects had sub-optimal vitamin A status, then additional vitamin A or β-carotene could positively affect hemoglobin incorporation of absorbed iron, iron transport

in the body and iron uptake by bone marrow, all of which are processes of iron metabolism that are impaired in iron deficiency (Walczyk *et al.* 2003). To further investigate this point, Davidsson *et al.* (2003) conducted a study in Côte d'Ivoire using children with low serum retinol concentration. In this study, children were fed a stiff maize porridge fortified with ferrous sulfate with or without the addition of retinyl palmitate. These authors found that vitamin A did not increase iron absorption, even in subjects with known vitamin A deficiency.

It was observed that the point of administration of the vitamin A compound was different between the two research groups and I hypothesized that this could have had an effect. Perhaps the introduction of the vitamin A compound prior to cooking would allow for more interaction time between the iron compound and retinyl palmitate or β-carotene, and perhaps that prevents the iron from interacting with other dietary components which may inhibit its dialyzability. However, the results presented here suggest that this does not explain the discrepancy because 1) the addition of vitamin A did not affect ferrous or total iron dialyzability as compared to the control, and 2) there was no difference in iron dialyzability whether the vitamin A compound was added before or after cooking.

3.14 Conclusions

The results of this investigation do not provide evidence for the previously reported claim that vitamin A and β-carotene increase iron absorption. All treatments demonstrated iron dialyzability similar to the control and were not statistically different.

THE ANIMAL STUDIES

CHAPTER 4: EFFECTS OF GREEN TEA ON IRON ABSORPTION AND THE SALIVARY PROTEOME IN RATS

4.1 Rationale

Tea has been shown to protect against cardiovascular disease (Kuriyama 2008; Velayutham *et al.* 2008), hyperglycemia (Venables *et al.* 2008), and some cancers (Butt & Sultan 2009). However, there is concern that tea drinking may negatively affect iron status. Considerable research has shown that drinking tea with an iron-containing food reduces nonheme iron absorption in both humans (Disler *et al.* 1975b; Samman *et al.* 2001) and rats (South *et al.* 1997; Hamdaoui *et al.* 2003). However, some data from rat studies suggest that the iron absorption inhibitory effects of tea disappear after 5 days of exposure to tea (Zhang *et al.* 1988; Kim & Miller 2005). This finding is supported by human epidemiological studies that report no association between tea consumption and iron status (Mennen *et al.* 2007; Hogenkamp *et al.* 2008). It has been suggested that increased secretion of proline-rich proteins (PRPs) into saliva could be a protective adaptation against the potential toxic and antinutritional effects of tea polyphenols (Kim & Miller 2005).

We hypothesized that exposure to dietary polyphenols increases the secretion of PRPs in saliva and that these PRPs bind to dietary polyphenols, reducing their capacity for iron binding, and that the inhibitory effects of tea on iron absorption will diminish over time due to the increased concentrations of PRPs in saliva. To test these hypotheses, we compared iron absorption and salivary protein composition in rats given short or long term exposure to tea, either by gavage or in the diet. We used weanling rats because we believed

their fast growth rate and high iron demands would make them more sensitive to changes in iron availability.

4.2 Materials and Methods

Chemicals. All chemicals were obtained from Sigma Chemicals (St. Louis, Mo., U.S.A.) or Fisher Scientific (Fair Lawn, N.J., U.S.A.) unless stated otherwise. Water used in the preparation of reagents was 18 MΩ ultra pure water. Glassware and utensils were soaked in 3 M HCl for no less than 4 hours and rinsed with de-ionized water prior to use. The stable isotope-labeled test meals were prepared by mixing aliquots of a ^{58}Fe solution containing 60 μg of ^{58}Fe (ferric) in dilute HCl (Isoflex USA, San Francisco, Calif., U.S.A.) with 2-gram portions of the basal (control) diet (20 mg Fe/kg diet, described in study design). Spray dried green tea extract was provided by Finlays (Florham Park, N.J., U.S.A.).

Method for determining iron binding polyphenols in green tea. The polyphenol content was measured using an iron-binding capacity assay (Brune *et al.* 1991), with tannic acid and catechin used as standards. The following solutions were prepared for the determination of polyphenol content in the tea: *(1) DMF-acetate (50% v/v).*—Prepared by mixing equal volumes of N,N'-dimethylformamide (DMF) and 0.1 M acetate buffer, pH 4.4. Solution was cooled to room temperature prior to use. *(2) Urea-acetate solution (50% w/v).*—500 g urea in 500 mL 0.1 M actetate buffer, pH 4.4. *(3) Ferric ammonium sulfate (FAS) solution.*—5% (wt/v) in 1 M HCl. *(4) Gum arabic solution.*—1% (wt/v) in ddH_2O. *(5) Iron-containing reagent.*—89 parts of 50% urea-acetate solution, 10 parts of 1% gum arabic solution, and 1 part of 5% FAS solution. Reagent prepared immediately before use. *(6) Food blank*

reagent.—89 parts of 50% urea-acetate solution, 10 parts of 1% gum arabic solution, and 1 part of 1 M HCl. Reagent prepared immediately before use. *(7) Standard solutions of tannic acid and catechin*.—Five dilutions at concentrations between 50 and 400 μg/mL were made with DMF-acetate.

Two mL of prepared green tea solution (0.3% green tea extract, by weight, in water) was diluted with 8 mL of DMF-acetate prior to analysis. 2 mL of the tea-reagent mixture was combined with 8 mL of FAS-reagent and after 15 minutes, the absorbance of the food sample was read at 578 nm and 680 nm versus a reagent blank consisting of 2 mL DMF-acetate and 8 mL FAS-reagent. The wavelengths correspond to the absorbance maxima of iron-galloyl (blue color; 578 nm) and iron-catechol (green color; 680 nm) complexes. After subtracting the absorbance of the food blank (2 mL of the tea-reagent mixture with 8 mL of the food blank reagent), the content of catechol groups (catechin equivalents) and galloyl groups (tannic acid equivalents) were calculated from standard curves. Absorbance measurements were made using a Beckman Coulter DU® 520 spectrophotometer.

Polyphenol concentration in the experimental green tea was 17% (wt/wt) tannic acid equivalents and 34% (wt/wt) catechin equivalents, on a dry weight basis.

Animal Diets. Diets and water were provided *ad libitum* throughout the study, unless specified otherwise. Three diets (Table 4-1) were prepared using a commercially prepared low iron, low polyphenol, semi-purified diet that was nutritionally complete except for iron (AIN-93G; Dyets, Inc., Bethlehem, Pa., U.S.A.). *Acclimation diet*: commercial diet containing $FeSO_4$ (ferrous sulfate) added at a rate of 25 mg Fe/kg diet. *Basal diet*: commercial diet containing $FeSO_4$ added at a rate of 20 mg Fe/kg diet. *Tea diet*: basal diet containing green

tea powder added at a rate of 28.6 g/kg diet. The tea-added animal feed was stored under refrigerated conditions throughout the study to minimize oxidation of polyphenols.

Animals and Study Design. Thirty-six weanling male Sprague-Dawley rats, with a mean body weight of 50 grams on arrival, were purchased from Charles River Laboratory (Wilmington, Mass., U.S.A.). The rats were housed individually in stainless steel cages with mesh bottoms. The cages were kept in a temperature-controlled room (at 22°C) with a 12-hour light/dark cycle. After arrival, the rats were given free access to the acclimation diet. At the end of the 7-day acclimation period, the animals were weighed and divided into 6 groups of 6 animals each so that the mean body weights in the groups were similar ($p=0.815$, data not shown). The groups were randomly allocated to the following treatments: acute control, acute gavage, acute oral, chronic control, chronic gavage and chronic oral. Control groups received the basal diet throughout the study. Gavage groups received the basal diet and were given a twice daily gavage of concentrated tea solution. This concentrated tea solution contained 0.25 g tea powder/mL H_2O and was prepared fresh every other day and stored in the refrigerator between gavages. The volume of tea given by gavage to the gavage groups was adjusted daily to reflect the amount of tea consumed in the diet by the oral groups. The oral groups were fed the green tea-supplemented basal diet and were given a twice daily sham gavage of phosphate buffered saline. The volume of saline given by gavage to the oral groups was adjusted daily to be equal to the amount of tea solution given by gavage to the gavage groups.

Table 4-1: Composition of experimental diets (DYET# 115072; Iron Deficient AIN-93G Purified Rodent Diet).

Ingredient	**Acclimation Diet (in g/kg diet)**	**Basal Diet (in g/kg diet)**	**Tea Diet (in g/kg diet)**
Casein	200	200	194
Sucrose	100	100	97
Cornstarch	397.461	397.466	386.556
Dyetrose	132	132	128
L-Cysteine	3	3	2.9
Cellulose (microcrystalline)	50	50	48.6
Soybean Oil	70	70	68
t-Butylhydroquinone	0.014	0.014	0.014
Mineral Mix #215009 (Rx, no Fe)	35	35	34
Vitamin Mix #310025	10	10	9.7
Choline Bitartrate	2.5	2.5	2.43
Iron (FeSO4)	0.025	0.02	0.02
Green Tea Extract	—	—	28.6
Total	**1000**	**1000**	**1000**

The stable isotope-labeled test meals were administered on day 1 (acute groups) and day 24 (chronic groups). Rats were fasted overnight prior to receiving the labeled test meal to ensure complete consumption of the 2 gram meal. After administration of the test meal, rats were allowed to consume their normal treatment diet *ad libitum*.

Animals were housed and tissues were collected at the East Campus Research Facility (Cornell University, Ithaca, N.Y., U.S.A.). The animal protocol was approved by the Cornell University Institutional Animal Care and Use Committee (Protocol 2007-0028).

Blood, Saliva and Liver Sample Collection. On day 1, a small amount of blood (around 20 μL) was collected from the lateral saphenous vein of all rats for baseline determination of $^{58}Fe/^{56}Fe$ ratios. Then labeled test meals were administered to the 3 acute groups. Seven days later, blood and saliva were collected from animals in the 3 acute groups. Ten minutes prior to collection, an intraperitonial (i.p.) injection of a ketamine solution (0.05-0.1 g/kg body weight) was used to anesthetize each rat. Blood was drawn using cardiac puncture for analysis of hemoglobin incorporation of ^{58}Fe. A pilocarpine solution (10 mg/kg body weight, dissolved in isotonic saline) was injected i.p. to stimulate salivary flow (Watson *et al.* 1989). Between 0.3 and 0.7 mL of saliva was collected as drool from each rat. Rats were then sacrificed by overexposure to CO_2. Whole liver was harvested for liver nonheme iron analysis. On day 24, the isotope test meals were given to the 3 chronic groups. Seven days later, blood, saliva and liver samples were collected following the procedure described above. The complete study design is summarized in Figure 4-1.

Iron Absorption. Blood was analyzed by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500 CS; Agilent Technologies, Santa Clara, Calif., U.S.A.) to determine

$^{58}Fe/^{56}Fe$ isotope ratios in hemoglobin iron. The total amount of excess ^{58}Fe in the circulating hemoglobin of each animal was calculated as follows (Abrams 1999):

$$^{58}\mathrm{Fe_{inc}}\ (\mathrm{mg}) = \frac{[^{58}\mathrm{Fe}/^{56}\mathrm{Fe}_{post} - {}^{58}\mathrm{Fe}/^{56}\mathrm{Fe_{pre}}] \times \mathrm{Fe_{circ}}\ (\mathrm{mg}) \times \mathrm{NA_{58}}}{^{58}\mathrm{Fe}/^{56}\mathrm{Fe_{pre}}}$$

where $^{58}Fe_{inc}$ (mg) is the mass in mg of the administered tracer present in the circulating hemoglobin; $^{58}Fe/^{56}Fe_{post}$ is the isotope ratio measured in circulating hemoglobin Fe 7 days following the administration of the tracer; $^{58}Fe/^{56}Fe_{pre}$ is the isotope ratio in circulating hemoglobin Fe just prior to the administration of the tracer; Fe_{circ}(mg) is the mass in mg of total circulating hemoglobin Fe; and NA_{58} is the natural abundance of ^{58}Fe (0.00287, mole fraction). Iron absorption was defined as the percentage of ingested ^{58}Fe present in circulating hemoglobin, assuming that 90% of the absorbed ^{58}Fe isotope was incorporated into circulating hemoglobin (Equation 1; Kastenmayer *et al.* 1994).

Liver Iron Stores. Nonheme iron concentrations in collected tissues were determined by the colorimetric method described by Schricker *et al.* (1983) as modified by Rhee & Ziprin (1987). *Solutions. (1) Acid mixture.*—6 M HCl and 40% trichloroacetic acid were mixed in equal volumes. *(2) Bathophenanthroline disulfonate reagent.*—0.162 g bathophenanthroline disulfonic acid sodium salt (BPDS) was dissolved in 100 mL water and 2 mL thioglycolic acid (96-99%) was added. The reagent was stored in an amber glass bottle. *(3) Saturated sodium acetate solution.*—400 g sodium acetate was stirred with 500 mL water. Once the

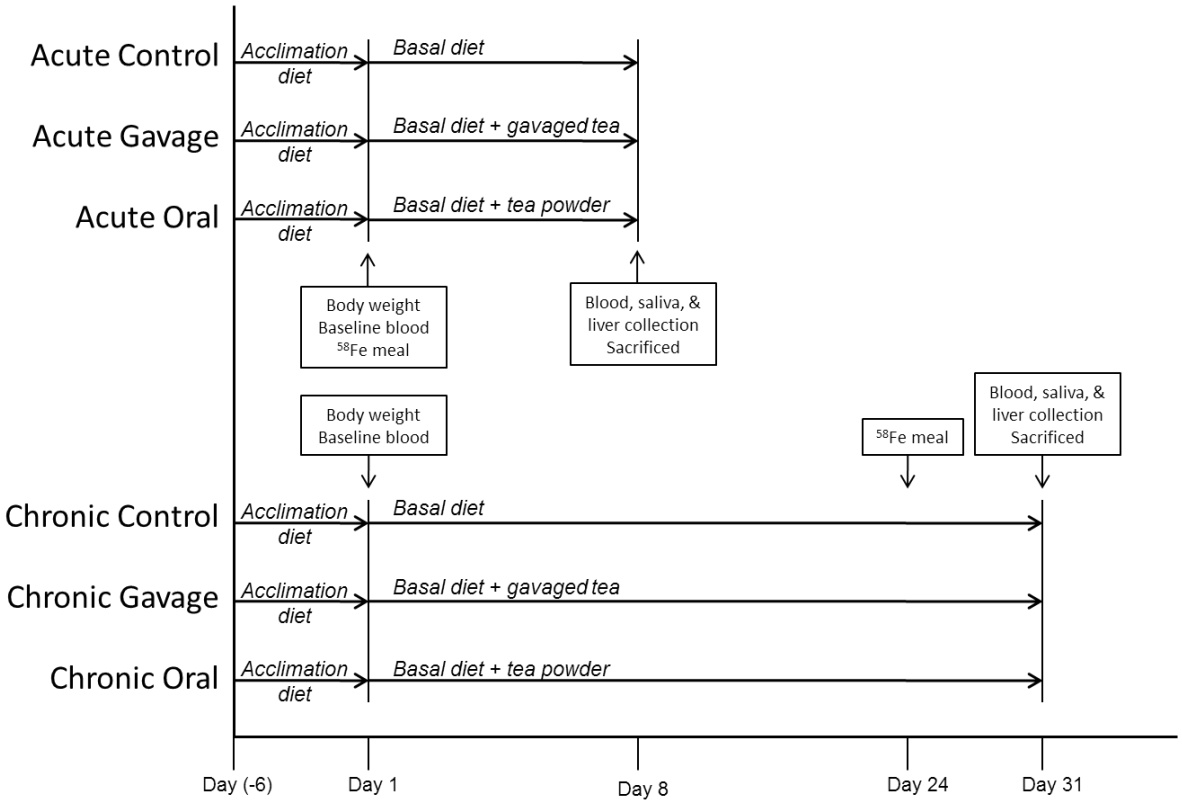


Figure 4-1: Study Design.

solution warmed to room temperature, more sodium acetate was added until no more sodium acetate would dissolve. *(3) Color reagent.*—20 parts of water, 20 parts of saturated sodium acetate solution and 1 part BPDS reagent. *(4) Color blank reagent.*—21 parts of water and 20 parts of saturated sodium acetate solution. *(5) $NaNO_2$ reagent.*—0.39% (wt/v) solution of $NaNO_2$ in water.

Five grams of finely chopped liver, in triplicate, were weighed into screw cap tubes and mixed thoroughly with 0.2 ml $NaNO_2$ reagent. The acid mixture (15 ml) was added to each tube and the tubes were vortex mixed thoroughly. Tubes were incubated in a water bath at 65°C for 20 hours and then cooled to room temperature. One ml of the acidic supernatant was transferred to a centrifuge tube and mixed with 5 ml of the color reagent. Samples were centrifuged at 3500 x g for 10 minutes. The absorbance of the supernatant was read at 540 nm against the reagent blank (1 ml acid mixture and 5 ml color reagent). A sample blank, consisting of 1 ml of the acid supernatant plus 5 ml of color blank reagent, was read at 540 nm against the regent blank (1 ml acid mixture and 5 ml color reagent). Results were expressed as micrograms of nonheme iron per gram of liver tissue, wet weight.

Qualitative Proteomic Analysis. Whole saliva was centrifuged for a total of 10 minutes at 16,000 x g to remove cellular debris and the supernatant combined 1:1 with an IEF (isoelectric focusing) solution consisting of 7 M urea, 2 M thiourea and 4% (wt/v) CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonium]-1-propanesulfonate). Protein concentrations in the diluted saliva samples were determined with the Bradford assay (Bradford 1976). The mean protein concentration of the diluted saliva samples was 3.4 ± 0.6 μg/μL (range: 2.6-5.0 μg/μL). Diluted saliva samples were stored at -80 °C until use. For analysis, aliquots of the

diluted saliva (from individual rats within a treatment group) were pooled such that the total amount of protein was 200 μg, with equal amounts of protein from each rat. Each pool was combined with DTT (dithiothreitol, 24 mM final concentration), IPG (immobilized pH gradient) solution (pH 3-10 nonlinear, 0.5% (v/v) final concentration, GE Healthcare) and enough IEF solution to produce a total volume of 250 μL. These final solutions were used to rehydrate 13 cm, pH 3-10 nonlinear IPG strips overnight prior to isoelectric focusing using an IPGphor II focusing unit (GE Healthcare Life Sciences, Piscataway, N.J., U.S.A.). 2D separation was performed using 12% SDS-PAGE gels on a SE 600 Ruby electrophoresis unit (GE Healthcare). Preparatory gels were stained using Colloidal Blue Staining Kit (Invitrogen, Carlsbad, Calif., U.S.A.). According to manufacturer's instructions, gels were fixed for 10 minutes in a 40% (v/v) methanol/10% (v/v) acetic acid solution prior to overnight staining with 200 mL of Colloidal Blue stain. Gels were destained in dH2O, with frequent changes, for 24 hours on a shaker table. These gels were used for tryptic digestions and peptide extractions (procedure detailed below).

To aid in the identification of PRPs, a second set of gels were run as described above and stained with 0.1% (wt/v) Coomassie Brilliant Blue R-250 (Invitrogen), for 3 hours in a 40% (v/v) ethanol/10% (v/v) acetic acid solution. With this procedure most proteins stain blue, but proline-rich proteins will stain pink (Beeley *et al.* 1991). These gels were destained in 10% (v/v) acetic acid for 24 hours at which point the pink colored proline rich protein bands could be clearly distinguished from the others. Pink colored gel spots, assumed to contain PRP's were excised and used for protein identification.

Tyrpsin Digestion and Protein Identification. Tryptic digestions and peptide extractions were performed following the protocol of Shevchenko *et al.* (1996) with slight modifications. Protein spots were primarily removed from gels stained with Colloidal Blue; additionally pink colored gel spots were recovered from gels stained with Coomassie R-250. Gel pieces were washed and destained using a wash consisting of nanopure water and 50% (v/v) acetonitrile/50% (v/v) 100 mM ammonium bicarbonate, and then dehydrated in 100% acetonitrile before drying. Once samples were completely dried, 0.2 μg of modified trypsin (Promega; Madison, Wis., U.S.A.) in 20 μL of 40 mM ammonium bicarbonate pH 7.8/10% (v/v) acetonitrile was added to each tube. Samples were left on ice for 15 min and incubated overnight at 30 °C.

The supernatant from each sample was recovered and the remaining peptides were then sequentially extracted from the gel using a series of washing solutions. The first contained 50 μL of 50% (v/v) acetonitrile with 2.5% (v/v) formic acid and the second 50 μL of 90% (v/v) acetonitrile with 0.1% (v/v) formic acid. The samples were sonicated for 10 min before removing the wash solution. The supernatants were combined with their respective washes and dried in a Speedvac Concentrator (Thermo Savant; Holbrook, N.Y., U.S.A.).

Samples were reconstituted for mass spectrometer analysis in 3 μL of 50% (v/v) acetonitrile (ACN) with 0.1% (v/v) trifluoroacetic acid (TFA). One μL of reconstituted sample was spotted on a MALDI target plate and allowed to dry. One μL of saturated matrix (10 mg/ml a-CHCA [a-cyano-4-hydroxy cinnamic acid] purchased from Sigma and crystallized prior to use in 50% (v/v) ACN with 0.1% (v/v) TFA and 1 mM ammonium phosphate) was spotted on top of the dried sample spots and allowed to dry completely.

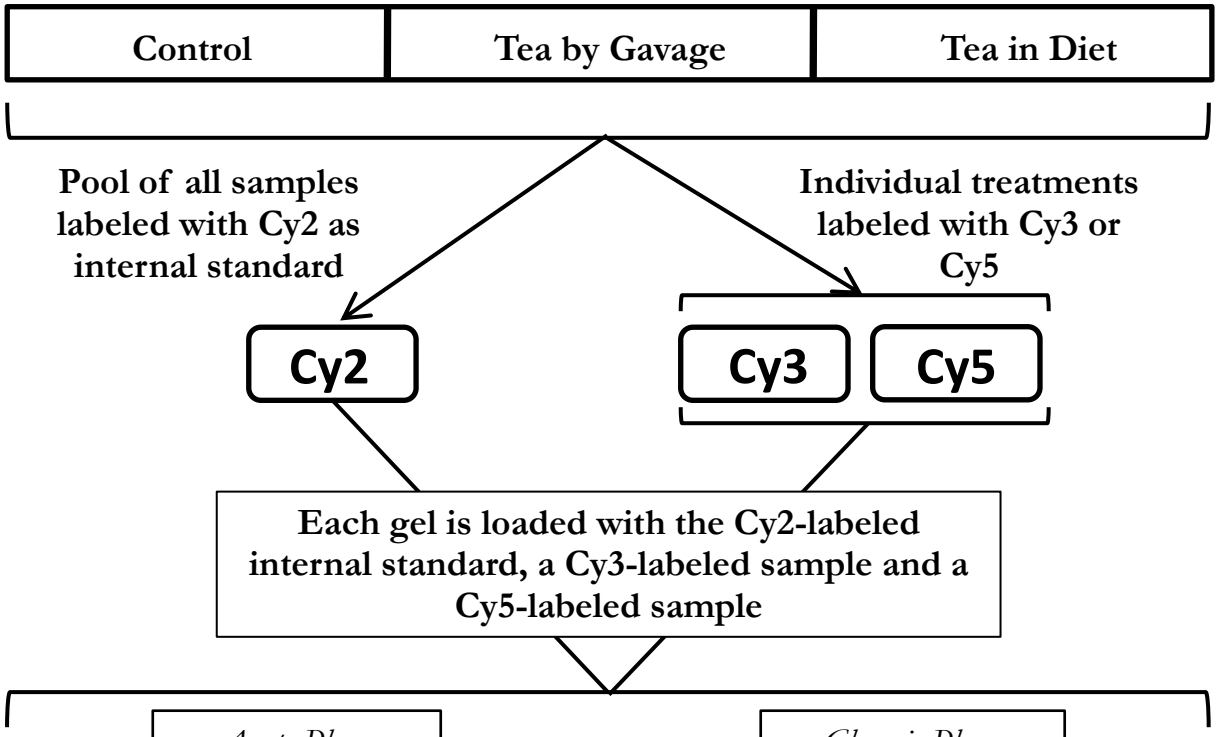
Samples were then subjected to matrix assisted laser desorption ionization (MALDI) MS/MS analysis using a 4700 Proteomics Analyser (Applied Biosystems; Framingham, Mass., U.S.A.) with 4700 Explorer version 3.6. This instrument was operated in 1 kV reflector positive ion mode and calibrated with a calibration kit (Applied Biosystems) containing a mixture of six standard peptides as a default calibration for spectra acquisition. Most samples were calibrated internally using three common trypsin autolysis products (at mass-to-charge ratio (m/z) values of 842.50, 1045.56, and 2211.09 Da) as mass calibrants. The external calibration was used as the default if the trypsin autolysis products were not observed in the spectra of the samples. The laser power was set to 4100 for MS and 5200 for MS/MS with CID off. MS spectra were acquired across the mass range of 800–4000 Da with a minimum S/N filter at 25 for precursor ion selection. MS/MS spectra were acquired for the 12 most abundant precursor ions with a total accumulation of 2400 laser shots per precursor.

The combined MS and MS/MS data from the MALDI TOF/ TOF analysis were submitted to Mascot 2.2 (Perkins *et al.* 1999) using GPS Explorer 3.6 for a search against the NCBInr *Rattus rattus*. The search parameters allowed for one missed tryptic cleavage, variable modification of methionine oxidation and fixed modification of cysteine carboxyamidomethylation. The precursor mass tolerance was 75 ppm and 0.25 Da was used for fragment ions. Only proteins with at least one peptide matching the target protein with >95% confidence interval (CI) were listed as identified.

Quantitative Proteomic Analysis. Difference gel electrophoresis (DIGE), which uses fluorescent dye labels, was used for quantification of the proteins (CyDye DIGE Fluors, GE

Healthcare). DIGE involves covalent labeling of two different protein extracts (e.g., from sample A and sample B) with one of two fluorescent cyanine dyes (typically Cy3 and Cy5). A third fluorescent dye (Cy2) is used to label a third protein sample (e.g., an equal mixture of A and B extracts) to provide an internal standard for sample normalization. The three labeled protein samples are then mixed and resolved on the same 2D gel. Since the dyes are all mass and charge matched, specific proteins from the different extracts migrate to the same 2D coordinate on the gel**.** The gel is scanned with a variable wavelength laser-based fluorescent imaging system. Since Cy2, Cy3, and Cy5 exhibit distinct excitation and emission spectra, it is possible to quantify and distinguish between proteins present in all three original extracts.

Dye swaps of Cy3 and Cy5 were used on all treatments to eliminate effects of dye bias. The acute DIGE experiment was run with 3 gels (2 technical replicates of each treatment) and the chronic experiment was run with 6 gels (4 technical replicates for each treatment). Samples were labeled according to manufacturer's instructions (GE Healthcare), such that each gel would contain 150 μg protein: 50 μg each of the Cy2-labeled internal standard, the Cy3-labeled sample and the Cy5-labeled sample. The labeled solutions were used to rehydrate pH 3-10 nonlinear IPG strips overnight prior to isoelectric focusing using an IPGphor II focusing unit (GE Healthcare). 2D separation was performed using 25 cm (acute) or 13 cm (chronic) 12% SDS-PAGE gels (after completion of the acute DIGE experiment, it was discovered that the smaller 13 cm gels were appropriate for the separation of these saliva samples). Gels were scanned on a Typhoon 9400 (GE) fluorescent scanner and images were analyzed using Progenesis SameSpots software (Nonlinear, Durham, N.C., U.S.A.). See Figure 4-2 for a schematic of the DIGE experimental design.



Gel	Cy2-label	Cy3-label	Cy5-label
1	Internal Standard	Control 1	Gavage 2
2	Internal Standard	Oral 1	Control 2
3	Internal Standard	Gavage 1	Oral 2

Technical pools were created from each treatment resulting in 2 pools for each treatment. These are indicated by number (1 or 2). The internal standard for each phase is composed of all six pools.

Gel	Cy2-label	Cy3-label	Cy5-label
1	Internal Standard	Control 1	Gavage 1
2	Internal Standard	Oral 1	Gavage 2
3	Internal Standard	Oral 2	Control 1
4	Internal Standard	Gavage 2	Oral 2
5	Internal Standard	Control 2	Oral 1
6	Internal Standard	Gavage 1	Control 2

Figure 4-2**:** DIGE experimental design. For DIGE analysis, *number of animals*=15 rats for acute phase (5 rats x 3 treatment groups) and *number of animals*=18 rats for chronic phase (6 rats x 3 treatment groups).

Statistical Analysis. For iron absorption, body weight and liver iron stores data, statistical analyses were performed using Minitab Release 14 and 15 (Minitab Inc., State College, Pa., U.S.A.). ANOVA was used to determine significant differences between means. Means were considered different at *p*-values less than 0.05. For statistical analysis of DIGE proteomics data, each gel was normalized using the pooled internal standard with the SameSpots software. One-way ANOVA analysis (with multiple testing correcting) was carried out to identify the protein spots whose change in intensity between treatment groups was statistically significant ($P \leq 0.05$). After identification of the protein spots by MS, data were combined so that all proteins for a particular family, for example amylase, were grouped. Protein group expression changes were calculated as weighted averages of normalized spot volumes reported in the Progenesis Samespots 2D analysis software.

4.3 Results

Body Weight. Tea feeding did not significantly affect body weight in the acute groups, $p=0.206$ (Table 1). In the chronic groups, both the gavage and oral groups had lower mean body weights than the control, but only the gavage mean body weight was significantly different from the control, $p=0.004$ (Table 1). Feed intake was also significantly lower in the chronic gavage groups than in the chronic control or chronic oral groups (Data not shown).

Liver Iron Stores. Liver iron stores were not significantly different among any of the six groups, $p=0.521$ (Table 1). The acute oral group had slightly depressed mean liver iron compared to the acute control or acute gavage, but this value was not statistically significant.

Iron Absorption from Labeled Meals. Iron absorption (Table 1) was not affected by the ingestion of tea in either the acute or chronic groups (p=0.104 and p=0.292, respectively). Overall, iron absorption was much higher in the acute groups than the chronic groups.

Table 4-2: Final body weight, liver iron stores and iron absorption comparisons among treatment groups (acute: day 8, chronic: day 31). Means ± standard error (SE), n=6 except where indicated by asterisk (n=5).

	Treatment	Acute	Chronic
Body weight (g)	Control	194.3 ± 11.6	384.1 ± 17.4 ^{B}
	Gavage	195.2 ± 5.1	308.4 ± 10.3 ^{A}
	Oral	176.7 ± 6.5	336.7 ± 11.4 ^{AB}
Liver iron stores (ug Fe/g liver)	Control	53.50 ± 11.10	60.97 ± 3.98
	Gavage	58.39 ± 1.99	57.62 ± 7.60
	Oral	47.21 ± 2.70	54.18 ± 7.47
Iron absorption (in %)	Control	43.13 ± 4.94*	5.62 ± 1.01
	Gavage	56.52 ± 7.52*	5.99 ± 0.97
	Oral	63.77 ± 6.36	3.70 ± 1.19

Qualitative Protein Differences in Saliva. SDS-PAGE of the saliva coupled with the Coomassie Brilliant Blue R-250 stain revealed an increase in acidic PRPs in the gavage groups when compared, qualitatively, to the control. The oral groups showed even more substantial increases in acidic PRPs when compared, qualitatively, to the control. These effects were observed in both the acute and chronic groups. Basic PRPs were detected in the acute control, acute gavage and acute oral groups, however, only the acute oral showed a substantial increase in basic PRPs when compared, qualitatively, to the control. Within the chronic groups, basic PRPs were only detected by the Coomassie R-250 stain in the chronic oral group. The PRPs are indicated by pink color and arrows in Figure 4-3 (acute phase) and Figure 4-4 (chronic phase).

Quantitative Protein Differences in Saliva. A summary of the protein expression differences revealed by DIGE analysis, shown as "fold change", is presented in Table 4-3. All protein expression changes shown in Table 4-3 are significant ($p<0.05$). Values should be compared within phases. Statistical comparisons were calculated within acute and within chronic groups, not between acute and chronic groups. A fold change of +2.6 indicates that the expression for the particular protein in the treatment group is 2.6 times higher than in the control group. A -8.0 value indicates that the treatment group protein expression is 8 times less, compared to the control. A fold change of 1.0 indicates that the expression is at the same level as that of the control.

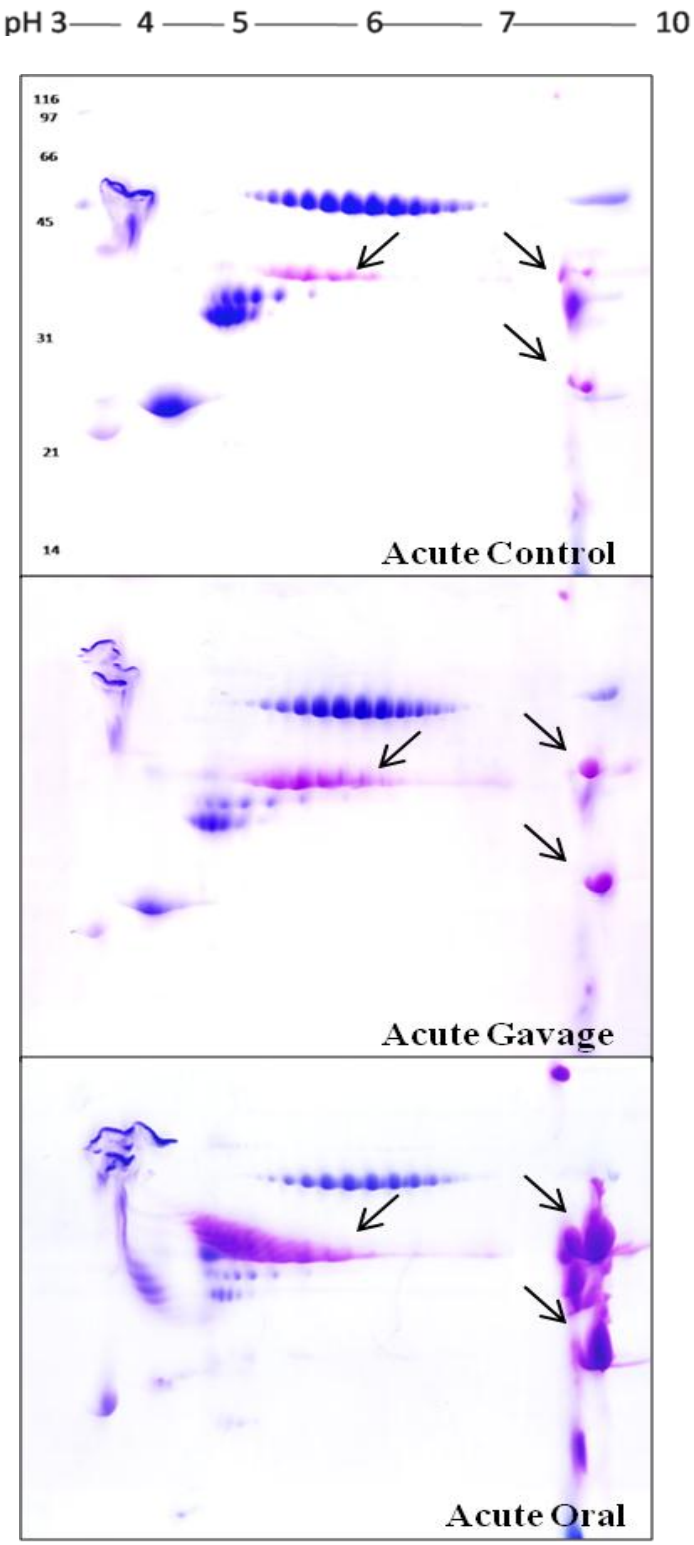


Figure 4-3: Acute phase 2D SDS-PAGE gels, pH 3-10 non linear, stained with Brilliant Blue R-250. Proline-rich proteins are indicated by arrows and pink color. The acidic PRP family and basic PRPs appear on all three gels: control (top), gavage (middle), and oral (bottom).

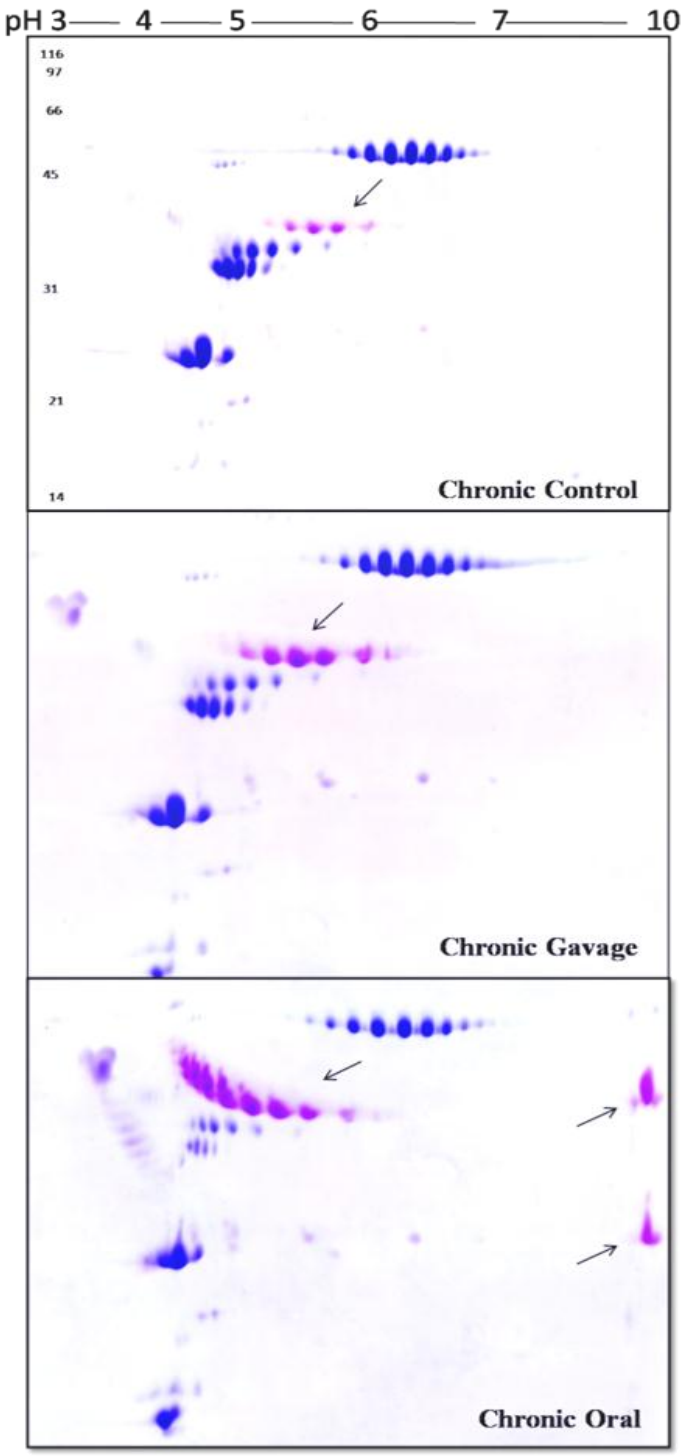


Figure 4-4: Chronic phase 2D SDS-PAGE gels, pH 3-10 non linear, stained with Brilliant Blue R-250. Proline rich proteins are indicated by arrows and pink color. The acidic PRP family appears on all three gels: control (top), gavage (middle), and oral (bottom). The remaining two PRPs appear only on the gel containing the oral treatment saliva (bottom).

Briefly, chitinase, deoxyribonuclease I, and cysteine-rich secretory protein 1, all decrease, compared to the control, and to a greater extent in the oral group than in the gavage group, within the same exposure time period (acute or chronic). Amylase and parotid secretory protein decreased compared to the control within the acute phase (oral group only) and chronic phase (oral and gavage groups), and to a greater extent in the chronic oral group than in the chronic gavage group. Submandibular gland secretory protein and the PRPs generally all increase to a greater extent in the oral group than in the gavage group, within the same exposure time period. Cystatin S increased to a greater extent in the chronic oral group than in the chronic gavage group.

Cystatin S was not detected in the acute phase DIGE gels. Cysteine-rich secretory protein 1 was significantly identified by MS/MS in the acute phase only. Protein "G", basic PRP "J" and basic PRP "K" are listed as not determined because they were not detected by the DIGE method in the saliva of acute gavage group. Both of the basic proline-rich proteins are described as "unidentified" due to the known difficulties of MS to significantly identify proline-rich proteins (Leymarie *et al.* 2002). However, these proteins were identified as PRPs using the Coomassie stain (Beeley *et al.* 1991). Protein "G" is described as "unidentified" because the confidence interval was not sufficient for significant identification.

Table 4-3**:** Relative expression of major salivary protein groups (as compared to the control). Spots are labeled on the gel map in Figure 4-5.

	Protein	Acute Phase		Chronic Phase	
		Gavage	Oral	Gavage	Oral
A	Amylase	1.0	-2.5	-1.2	-1.5
B	Chitinase	-2.3	-6.4	-1.3	-2.1
C	Unidentified acidic PRP	+2.2	+8.3	+2.1	+5.6
D	Deoxyribonuclease I	-1.4	-3.2	-1.8	-2.4
E	Cysteine-rich secretory protein 1 [a]	-1.8	-8.0	-1.6	-5.7
F	Submandibular gland secretory protein	+1.8	+3.1	+2.0	+4.0
G	Unidentified protein	ND	+3.1	+1.7	+23.3
H	Parotid secretory protein	1.0	-5.0	-1.3	-1.6
I	Cystatin S	ND	ND	+19.5	+57.5
J	Unidentified basic PRP	ND	+13.3	+2.9	+4.1
K	Unidentified basic PRP	ND	+8.1	+2.2	+2.6

[a]This protein was not significantly identified by MS/MS analysis in the chronic phase; ND=Not determined.

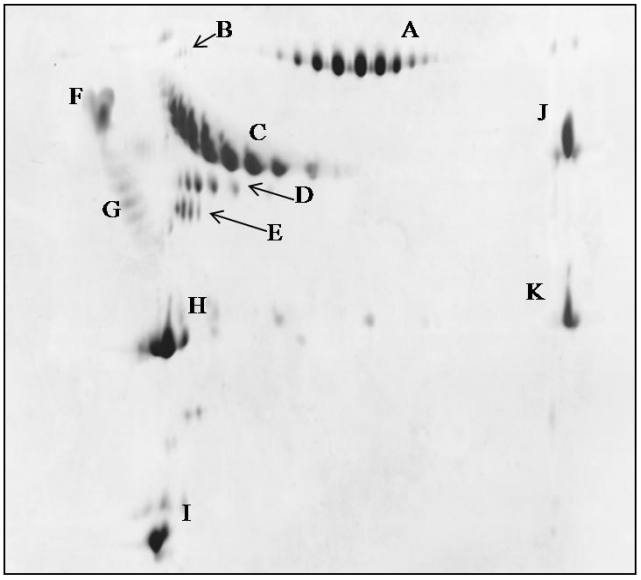


Figure 4-5: Labeling key for Table 4-3. Black and white rendition of the chronic phase oral group 2D SDS-PAGE gels, pH 3-10 non linear, stained with Brilliant Blue R-250.

A summary of the protein identification scores are shown in Table 4-4. All proteins identified in table were considered significant (protein and total ion confidence interval percentages > 95%). A significant finding indicates that the protein was identified in that spot. A complete list of scores for all identified spots is presented in Appendix A. The accession number (Acc. No.) is a unique identifier of individual proteins within the database NCBInr *Rattus rattus*. Protein score, total ion score and best ion score were based on the Mascot search. The protein score confidence interval (C.I.), total ion score C.I. and best ion score C.I. were calculated by GPS Explorer. The *ion score* and *protein score* are based on the probability that the observed match between the experimental data and the database sequence is a random event. For the best ion score, the threshold of identity for the peptides at the 95% confidence level was 52. Best ion scores above this value were considered significant. For the protein scores, the threshold of identity at the 95% confidence level was 81 and protein scores above this value were considered significant. The *total ion score* is the sum of the highest ions score for each distinct sequence matching the protein (excluding duplicate matches). The high values for the *protein score confidence interval (C.I.), total ion score C.I.* and *best ion score C.I.* suggest that the confidence in the identifications presented was considerably greater than 95%. *Pep count* is the number of peptides matched in MS/MS analysis for the identification of the protein.

The confidence interval was too low for positive identification of cysteine-rich secretory protein in the chronic phase and cystatin S was not detected in the acute phase. Therefore these data are not shown in Table 4-4.

Table 4-4**:** Protein identification parameters. Summary data based on a search against the NCBInr database *Rattus rattus*. All proteins identified in table were considered significant.

Protein	Acc. No.	Protein score	Pep count	Protein C.I. %	Total Ion score	Total ion C.I.%	Best Ion score	Best Ion C.I.%
Amylase (acute)	gi|56971297	354	21	100	145	100	58	99.9
(chronic)	gi|56971297	809	18	100	565	100	84	100
Chitinase (acute)	gi|46485462	150	8	100	89	100	52	99.8
(chronic)	gi|46485462	231	9	100	117	100	60	99.9
Deoxyribonuclease I (acute)	gi|6978769	384	9	100	284	100	62	100
(chronic)	gi|149042674	344	7	100	276	100	92	100
Cysteine-rich secretory protein 1 (acute)	gi|12408314	240	10	100	147	100	57	99.7
Submandibular gland secretory protein (acute)	gi|204477	112	2	100	104	100	76	100
(chronic)	gi|204477	160	2	100	153	100	92	100
Parotid secretory protein (acute)	gi|16258825	335	7	100	256	100	77	100
(chronic)	gi|16258825	217	3	100	193	100	112	100
Cystatin S (chronic)	gi|149030955	301	3	100	270	100	164	100

4.4 Discussion

Younger rats (acute phase) absorbed more of the ^{58}Fe than older rats (chronic phase). This finding is in accordance with previous research using rats which demonstrated that age plays a role in iron absorption (Forbes & Reina 1972).

We expected that the rats receiving tea would have significantly lower iron absorption than the control (Zhang *et al.* 1988; South *et al.* 1997). A previous study from our lab showed rats given iron mixed with a black tea infusion absorbed less iron than rats given iron dissolved in water (Kim & Miller 2005). However, our results in the present study show no significant effect of tea on iron absorption. A possible reason for this discrepancy is that in the previous study, the tea and iron were administered together. Mixing the tea with the iron prior to administration may have allowed for more interaction between the tea polyphenols and iron, which could explain the observed inhibitory effect of the tea on iron absorption. In the current study, iron and tea were not mixed prior to administration; instead iron was administered in a test meal, immediately followed by tea consumption in the diet or by gavage. However, since the iron test meal and tea were given within a short time, it was expected that the tea and iron would have had time to interact prior to reaching the intestinal absorption site. Another possible reason that we did not observe an iron inhibitory effect but others did was that green tea was used in the present study whereas black tea was used in the earlier study. However, Hamdaoui *et al.* (2003) reported that a green tea decoction (boiled tea drink) inhibited iron absorption in rats by the same amount as a black tea decoction. Similarly, Samman *et al.* (2001) showed that the addition of pressed leaf green tea extract to a meal decreased nonheme iron absorption in women.

While there was no observed effect on iron absorption, green tea did cause marked changes in proline-rich protein expression, in agreement with previously reported work (Kim & Miller 2005). The observation that chronic tea exposure by gavage increased PRP expression indicates that stimulation of salivary PRP secretion occurs as a result of some systemic signal. In the gavage groups, the tea is delivered directly into the stomach, bypassing the oral cavity. Rats are unable to regurgitate any food or liquids (Montedonico *et al.* 1999), therefore, the observation that PRP expression was increased in the rats given tea by gavage suggests that either absorbed polyphenols circulating in the blood stimulate PRP expression or that a neural or hormonal signal originating in the stomach or small intestine influences PRP expression in the salivary glands. However, since the animals receiving tea in their feed produced substantially higher amounts of PRPs than either the gavage or control groups, local signals are likely the predominate mechanism for this effect. To our knowledge, this is the first report of a comparison of PRP expressions following oral and gavage exposure to tea.

The increased expression of PRPs in the saliva of the rats fed tea but not in the rats fed the control diet supports earlier work that in the presence of polyphenols, rats will increase their PRP secretion. If this same process occurs in humans it could explain why habitual tea drinking has not been shown to be associated with iron deficiency (Mennen *et al.* 2007). Nelson and Poulter (2004) reviewed 12 observational studies and reported that after accounting for other dietary factors, tea drinking alone does not increase one's risk for iron deficiency in healthy individuals. Hogenkamp *et al.* (2008) studied black African adults and reported that tea consumption in a subpopulation considered at risk for iron deficiency was

not correlated with iron status. Additionally, Mehta *et al.* (1992), using data from the second National Health and Nutrition Examination survey, found the risk of iron deficiency was slightly lower in tea drinkers compared to non-tea drinkers. While there are data indicating that tea consumption decreases iron absorption from a single meal, the observational studies mentioned, and others, suggest that humans and rats have mechanisms to overcome the suppression of iron absorption by tea.

While our main focus was on PRP expression in salivary glands, the changes in other proteins were also of interest. In addition to PRPs, we detected amylase, chitinase, cystatin S, deoxyribonuclease1 (DNase1), cysteine-rich secretory protein 1, submandibular gland secretory protein and parotid secretory protein. Three of these proteins—amylase, chitinase and DNase1—are enzymes. Amylase cleaves the α-1,4-linkages of amylose (Gray 1992); while DNase1 cleaves DNA endonucleolytically resulting in singular and oligo-nucleotides (Kishi *et al.* 2001). Chitinase breaks down chitin, which is abundant in the cell walls of fungi and the exoskeletons of insects. It is believed that chitinase in the saliva may provide a defense mechanism against pathogens containing chitin in their cell walls (Flach *et al.* 1992).

Of the remaining proteins we found in the rat saliva, two of these proteins—the submandibular secretory protein and the parotid secretory protein (PSP)—are closely related proteins with high leucine content and unknown functions, although it has been suggested that PSP may have anticandidal and antibacterial properties (Gupta *et al.* 2000; Khovidhunkit *et al.* 2005). Cysteine-rich secretory protein 1 is produced in both the saliva and in the reproductive tract (Roberts *et al.* 2006). Cystatin S is a cysteine protease inhibitor.

It is significant that all proteins did not increase or decrease to the same extent, indicating that the rat responds to the tea stimulus by making protein-specific changes. These increases and decreases were present in both the oral and the gavage groups of the two phases, and while the direction of change was consistent between groups, the extent of the increase or decrease was generally smaller in the gavage groups than in the respective oral groups.

While it is unknown what role some of these proteins may play, the findings here support current observations that at least some salivary proteins may help protect the body from harmful dietary polyphenols. It has been observed that capsaicin, an irritating substance, induces salivary cystatin expression in rats (Katsukawa *et al.* 2002), leading to the belief that cystatins not only inhibit ingested cysteine proteases, but also provide protection from toxic or irritating dietary compounds (Dickinson 2002). It has been suggested that cystatins may be able to bind to dietary polyphenols, thereby reducing their antinutritional effect (Mau *et al.* 2011). Recently it has been reported that chitinase expression decreases in response to polyphenol feeding in mice (Lamy *et al.* 2010), which agrees with the findings we present here. While the main function of chitinase is believed to be protection from chitin-containing pathogens, it may have a role in protecting against adverse effects of polyphenols as well. Parotid secretory protein has been shown to decrease when proline-rich proteins are stimulated using isoproterenol (Ann *et al.* 1987). It has been reported that α-amylase expression increases in response to dietary polyphenols, possibly due to polyphenol inhibition of amylase activity (da Costa *et al.* 2008; Lamy *et al.* 2010). Our study is not in agreement with these findings as we found amylase decreased in response to green tea

feeding. More research is needed to discover the roles these proteins, including amylase, play in protecting the body from dietary polyphenols.

4.5 Conclusion

Our results suggest that while iron absorption and liver iron stores may not be affected by green tea consumption, the polyphenols in green tea stimulate dramatic changes in the expressions of various salivary proteins in the rat, including markedly increased proline-rich protein secretion. The presence of increased proline-rich proteins in the gavage groups indicates the possibility of a systemic effect, or secondary stimulation site, in addition to the local effect in the mouth.

CHAPTER 5: EFFECTS OF GREEN & BLACK TEA ON IRON ABSORPTION & THE SALIVARY PROTEOME IN PIGLETS

5.1 Rationale

In the previous experiment, a rat model was utilized to investigate the effect of tea consumption on iron absorption and the profile of salivary proteins. Unlike other published findings, it was observed that green tea ingestion did not decrease iron absorption in the rat, whether the tea was mixed into the diet or given by gavage. However, similar to other published findings, it was demonstrated that even the control rats secreted small amounts of PRPs, but the rats fed tea significantly increased the expression of salivary PRPs in response to the tea feeding. This finding was significant because it implies that salivary PRPs play a major role in protecting the body against the detrimental effects of iron-binding polyphenols. However, while rats are excellent models due to their wide availability, small size and inexpensive cost, they may respond differently to tea polyphenols than humans. In the experiment presented here, the piglet was used as a model. The physiological and anatomical similarities of the digestive tract of the pig to man makes the pig an important and useful model (Patterson *et al.* 2008).

5.2 Objective

The objective of the experiments described in this chapter were to test the hypothesis that green or black tea ingestion would not decrease iron absorption, but would increase secretion of PRPs in whole saliva and increase mRNA expression of PRP genes in the

parotid gland. A further objective was to observe the effects of tea consumption on the gut microbial community.

5.3 Approach

Two experiments were run to investigate the effects of tea consumption in a piglet model. In experiment 1, animals were housed in individual pens, so that individual iron intake could be monitored. Saliva was collected weekly to monitor changes in salivary protein expression throughout the experiment. In experiment 2, animals received tylosin (an antibiotic) in their feed to help combat the high incidence of diarrhea observed in experiment 1, and animals were housed together in group pens instead of in individual pens. At the conclusion of experiment 2, animals were sacrificed and their tissues collected for analysis.

5.4 Materials & Methods

All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee (Protocol 2005-0084).

Chemicals. All chemicals were obtained from Sigma Chemicals or Fisher Scientific unless stated otherwise. Water used in the preparation of reagents was double deionized. Glassware and utensils were soaked in 3 M HCl for no less than 4 hours and rinsed with deionized water prior to use. Spray dried green tea extract and black tea extract were sourced from Finlays (Florham Park, N.J., U.S.A.). The polyphenol content was measured using an

iron-binding capacity assay (Brune *et al.* 1991), with tannic acid and catechin used as standards. This assay was previously described in Section 4.2. The polyphenol concentration in the experimental green tea was 14% and 38% (by weight) tannic acid and catechin equivalents, respectively, on a dry weight basis. The polyphenol concentration in the experimental black tea was 10% and 24% (by weight) tannic acid and catechin equivalents, respectively, on a dry weight basis.

Animal Diets. Diets were formulated to meet the National Research Council nutrient recommendations for piglets (NRC 1998), except for iron. Vitamin/mineral premixes were prepared in the lab using reagent grade chemicals (Table 5-1). Whole dent corn was ground and mixed thoroughly with whole soybean meal before adding the other ingredients (Table 5-2). To prepare the tea diets, 1% (wt/wt) green tea extract or black tea extract was mixed with the basal diet. The respective basal diet was given to the weaned piglets until the start of the experiments. Iron content of all diets was 45± 3 mg/kg diet, as determined by ICP-AES after wet ashing. Diets and water were provided *ad libitum* throughout the study, unless specified otherwise.

Piglets. Piglets were farrowed and housed at the Cornell University Swine Research Farm. Newborn crossbred piglets (Yorkshire × Hampshire × Landrace) were given an intramuscular injection of iron dextran (Sanofi Animal Health Inc., Overland Park, KS) containing 50 mg of Fe, half of the recommended dose, so that they would develop iron deficiency prior to weaning. Piglets were weaned around 21 days of age and fed the iron-deficient basal diet until beginning their treatment diet.

Table 5-1. Composition of the vitamin/mineral premix, expressed as the concentration in the diet.

Vitamins/Minerals	**Concentration in Diet (per kg diet)**	
Vitamin A (retinyl palmitate)	2200	IU
Vitamin D (ergocalciferol)	250	IU
Vitamin E (dl-α-tocopherylacetate)	16	IU
Vitamin K	0.63	mg
Biotin	1.25	mg
Choline	830.00	mg
Folacin	0.38	mg
Niacin	15.15	mg
Panthothnic acid	10.00	mg
Riboflavin	4.38	mg
Thiamin	1.02	mg
Vitamin B6	1.52	mg
Vitamin B12	1.75	mg
Copper (copper sulfate)	24.00	mg
Selenium	7.50	mg
Iodine (potassium iodide)	0.20	mg
Zinc (zinc oxide)	170.94	mg
Manganese (manganese sulfate)	13.56	mg
Iron	0.00	mg

Table 5-2. Composition of the experimental diets

Ingredient	Experiment 1 % of diet	Experiment 2 % of diet
Plasma, spray dried	1.00	1.00
Corn	69.15	68.79
Soybean meal (48%)	24.00	23.88
Corn oil	1.00	1.00
Lysine HCl	0.25	0.25
Theronine	0.10	0.10
Methionine	0.10	0.10
Sodium Chloride	0.25	0.25
Sodium Phosphate	1.30	1.29
Calcium Carbonate	1.60	1.59
Vitamin/Mineral Premix	1.00	1.00
Magnesium Oxide	0.25	0.25
Tylosin	0.00	0.50
Total	**100**	**100**

EXPERIMENT 1

Study Design. Prior to weaning, piglets were given a commercial creep feed. After weaning, piglets were given the iron-deficient control diet. Seven days prior to the start of the experiment, 33 piglets (~28 days old) were weighed and divided into groups so that mean body weights were similar and littermate and gender distribution between the groups was equal. The piglets were moved to the Block Barn at the Swine Research Farm, housed individually in caged pens with concrete floors (approximately 1m x 4m in area) in a barn maintained between 22- 30°C, with a 12 h light/dark cycle. Piglets were allowed to acclimate to their new environment for 7 days prior to the start of the treatment diet (at age 35 days). The groups were randomly assigned to either the basal diet (Control), green tea diet (Green) or black tea diet (Black).

Feed was weighed before administration to each piglet's feeder and leftover and spilled feed were collected and weighed daily. Body weight measurements, blood and saliva collection were conducted once a week for the duration of the study. Feed was withdrawn from the piglets 12 hours before saliva collection and weight measurement. The study design for Experiment 1 is presented in Figure 5-1.

EXPERIMENT 2

Study Design. Prior to weaning, piglets were given the iron-deficient control diet as creep feed. After weaning, piglets continued receiving the iron-deficient control diet. Seven days prior to the start of the experiment, 36 piglets (~24 days old) were weighed and divided

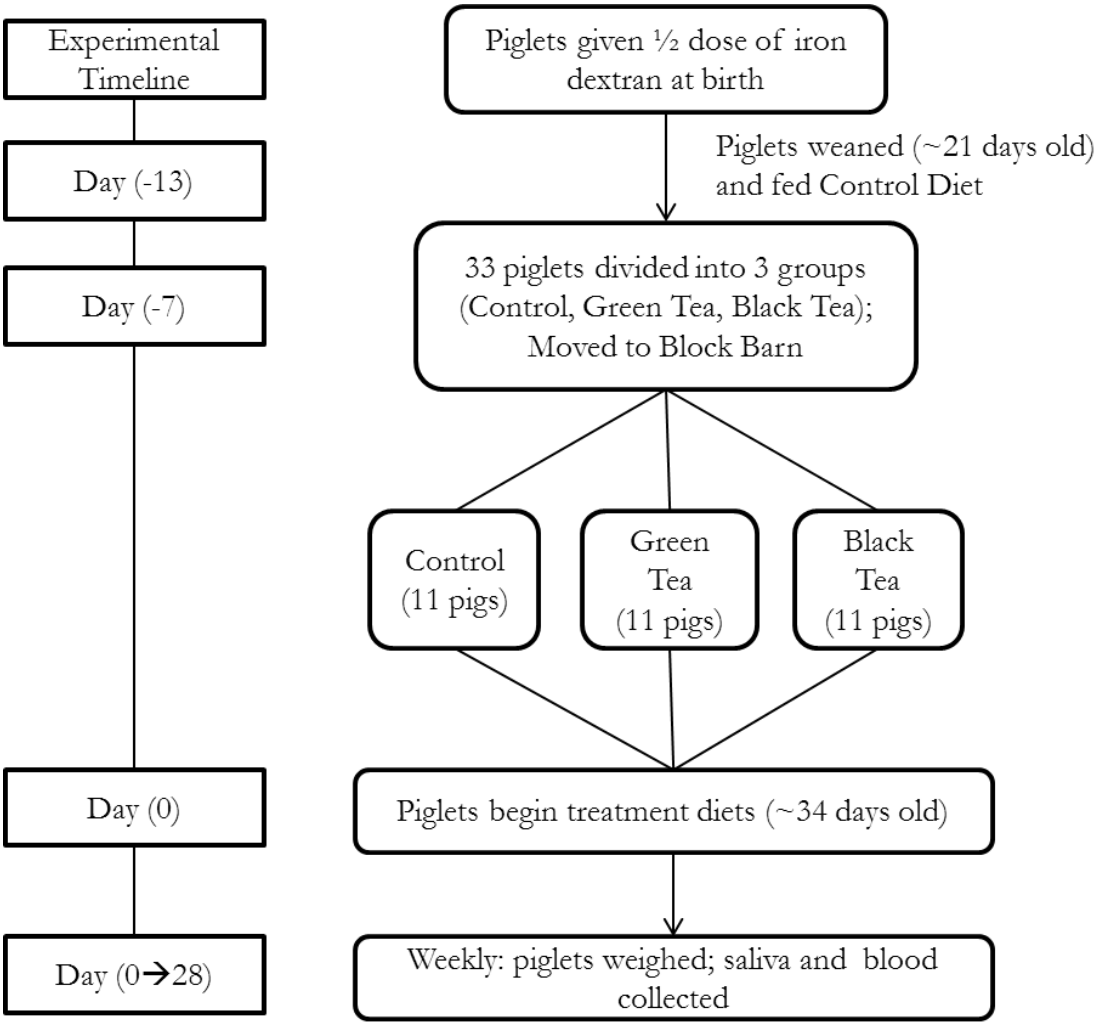


Figure 5-1: Study design for Experiment 1

into groups so that mean body weights were similar and littermate and gender distribution between the groups was equal. The groups were randomly assigned to either the basal diet (Control), green tea diet (Green) or black tea diet (Black). The piglets were moved to 3 group pens, such that all pigs in a group (Control, Green, Black) shared one pen. The animals were allowed to acclimate to their new environment for 7 days prior to beginning the experimental diets (at age 35 days).

On the first day of the experiment, two piglets, that were also fed the iron-deficient basal diet since weaning but were not assigned to any group, were slaughtered as Day 0 controls.

After two weeks of the experiment, the piglets were moved to 6 group pens, such that the 5 largest piglets of a group shared one pen and the 5 smallest piglets of a group shared one pen. The animals were separated this way because the larger animals were better competitors for food than the smaller animals. The group pens were housed in the main building of the Swine Research Farm, which was maintained between 22-25°C, with a 12 h light/dark cycle. Feed was weighed before placing in the group feeders and leftover feed was collected and weighed. However, due to the nature of the group pens, it was not possible to recover spilled feed. Body weight measurements and blood collections were conducted once a week for the duration of the study.

On the final day of the experiment (Day 35), saliva was collected from all animals in addition to the regular weighing and blood collection. The 5 largest animals per group were given their respective diets until they were slaughtered, 2 days later. Feed was withdrawn 8 h before slaughter and the animals were killed by electrical stunning and exsanguination. The parotid glands (left and right) were excised immediately, frozen in liquid nitrogen and stored

at -80°C until analysis. Liver samples were excised and frozen in liquid nitrogen prior to storage at -80°C.

Segments were excised from the cecum (excised at the distal end of the cecum), and the mid-colon (excised from where the spiral colon changes direction from spiraling downwards to spiraling upwards), and the segments were cut longitudinally to expose the mucosa. Samples were placed in wash tubes containing 25 mL of ice-cold brain heart infusion broth (BHIB) containing 20% (v/v) glycerol. The samples were stored on ice until completion of the slaughter. In the lab, tubes were agitated on ice to remove luminal bacterial and digesta. The tissue was carefully dried using a sterile paper towel and remaining digesta gently scraped away with sterile scissors. The segment was transferred to a detergent wash tube, containing a pH buffered solution (pH 7.6) of 25 mM Tris-HCl, 1% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulfate, 1% (w/v) sodium deoxycholate and 10 mM EDTA. Adherent bacteria were released by gentle agitation of the tubes on ice for 30 minutes. Tissues were removed from the wash tubes and the tubes were centrifuged at 12,000 x g for 5 minutes. The supernatant was removed and the pellet was re-suspended in 5 mL BHIB/20% glycerol solution and stored at -80°C until microbial analyses were performed using terminal restriction fragment length polymorphism (TRFLP) analysis. The above method is similar to a procedure which has been previously described (Patterson *et al.* 2005).

The study design for Experiment 2 is presented in Figure 5-2.

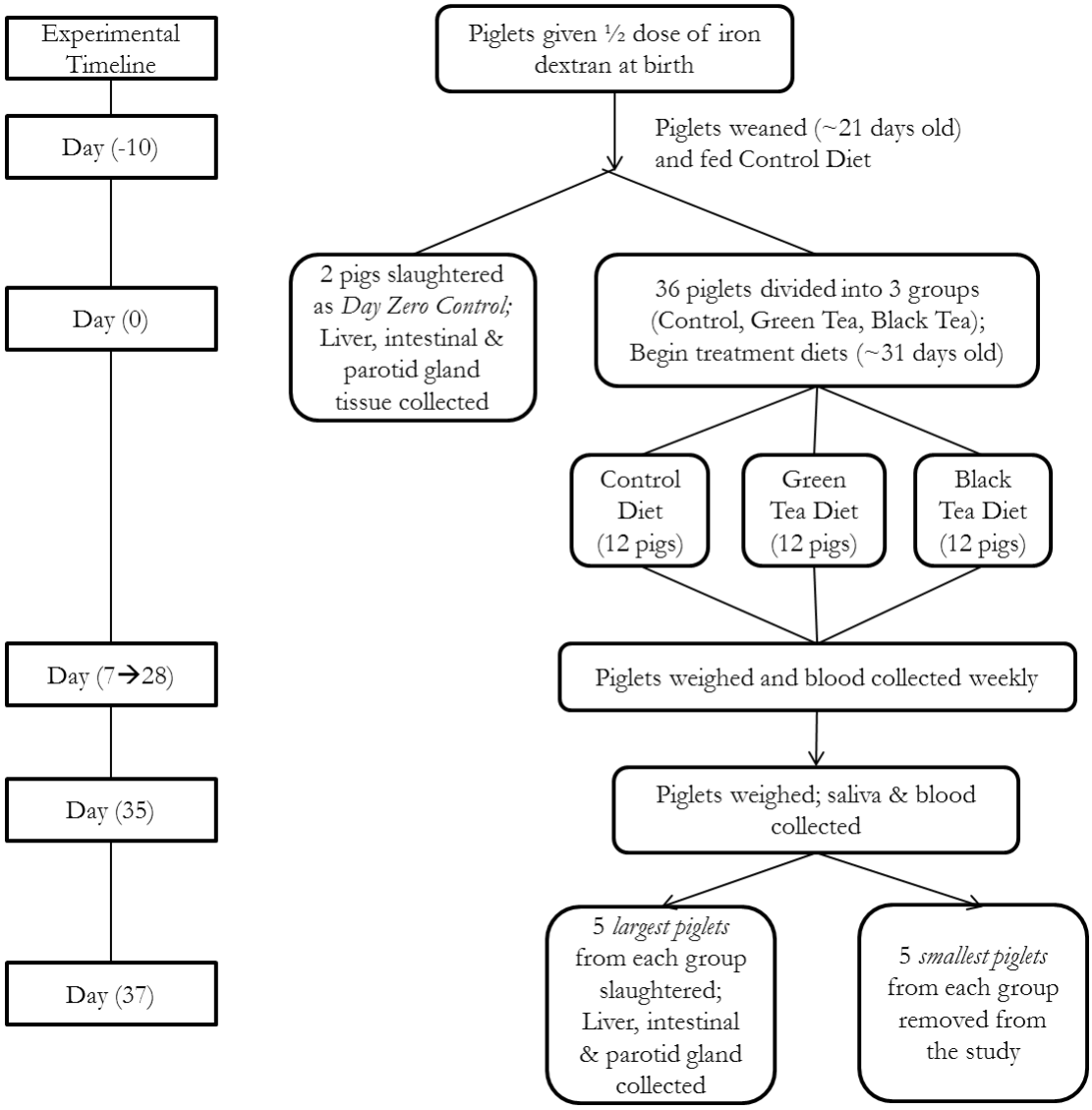


Figure 5-2: Study design for Experiment 2

Sample Collection

Blood collection. Blood samples were drawn via jugular venipuncture into Vacutainer tubes containing sodium heparin (Becton, Dickinson and Co; Franklin Lakes, NJ, USA), which prevents clotting. Immediately after collection, tubes were stored on ice and hemoglobin concentrations were analyzed within 4 hours of collection. After determination of hemoglobin concentration, samples were centrifuged at 600 x g at 4°C for 15 minutes to pack the red blood cells at the bottom of the tube without lysing them. The clear supernatant (blood plasma) was removed and stored at -80°C until analysis.

Saliva collection. A previously described method (Sorrells *et al.* 2006) was utilized to collect saliva from each piglet. One mL of 5% (w/v) citric acid solution was administered to each piglet's tongue to induce salivation, and then the piglets were allowed to chew on a cotton roll (Salivette® Tube w/out Preparation; Sarstedt; Nümbrecht, Germany) secured with fishing line until the roll was wet or 2 minutes had passed. The cotton rolls were placed into pierced tubes fitted inside specially designed collection tubes. Tubes were stored on ice for transport to the laboratory, where tubes were immediately centrifuged at 3,000 x g at 4°C for 7 minutes. This forced the saliva in the cotton swabs to the bottom of the collection tubes. Saliva was removed from the collection tube and stored at -80°C until analysis.

Sample Analysis

Hemoglobin repletion efficiency assay. Hemoglobin concentrations were determined colorimetrically by the cyanmethemoglobin method using a kit (Pointe Scientific Inc, Canton, MI, USA). According to manufacturer's instructions, Drabkin's Reagent was

prepared by combining 1 vial of Drabkin's Reagent powder with 900 mL of ddH_2O and 0.5 mL Brij 35 solution, the resulting mixture was brought to 1000 mL with ddH_2O. 8 μL of each blood sample, in triplicate, were added to individual, disposable glass tubes containing 2 mL of Drabkin's Reagent. Solutions were vortex mixed and then allowed to sit at room temperature for 15 minutes and then were read at 540 nm using a spectrophotometer. Hemoglobin concentrations were determined using a standard curve generated with a reconstituted hemoglobin standard (Pointe Scientific, Inc).

Total body hemoglobin iron for each pig was calculated from the hemoglobin concentration and body weight according to the following formula (Tan *et al.* 2008):

$$\text{Hb Fe(mg)} = [\text{BW (kg)} \times 0.067 \text{ L of blood/kg of BW}] \times [\text{Hb (g/L of blood)}] \times [3.35 \text{ mg of Fe/g of Hb}]$$

where Hb was hemoglobin concentration, Hb Fe was the total body hemoglobin iron, BW was the piglet's body weight and blood volume, in L, was assumed to be 6.7% of the body weight, in kg. Hemoglobin repletion efficiency (HRE) was calculated according to the following formula (South *et al.* 2000):

$$\text{HRE} = \frac{\text{Hb Fe, mg (final)} - \text{Hb Fe, mg (initial)}}{\text{total Fe intake (mg)}} \times 100$$

where final Hb Fe was taken as the value calculated for days 7 (Week 1), 14 (Week 2), 21 (Week 3), or 28 (Week 4), and initial Hb Fe was taken as the value calculated for the previous data point. HRE is an estimate of the percentage of ingested iron that is absorbed

and utilized. As HRE only takes into account the amount of iron that utilized in hemoglobin, but does not account for the approximately 10% of absorbed iron that is utilized in myoglobin or other proteins (Tan *et al.* 2008), it is a small underestimate of the actual % of iron absorbed.

Plasma iron concentration. A modified colorimetric iron assay was used to calculate plasma iron concentration (Bothwell 1971). The following solutions were prepared for the determination of plasma iron concentration: *(1) Chromagen solution.*—sodium acetate (1.5 M) containing 5.3 mg/mL BPDS (4,7-diphenyl-1,10-phenthroline disulfonic disodium salt). *(2) Sodium acetate buffer.*—sodium acetate (2.0 M) in ddH_2O. *(3) Protein precipitant solution.*—10% (w/v) trichloroacetic acid, 5% (w/v) hydroxylamine monohydrochloride, and 10% (v/v) concentrated HCl in ddH_2O.

One mL aliquots of thawed plasma, in duplicate, were transferred into labeled disposable glass tubes containing 1 mL of protein precipitant solution and vortex mixed. Immediately, the proteins began to precipitate and after 15 minutes, approximately 1.75 mL of the liquid was removed and placed into 2 mL centrifuge tubes. The samples were centrifuged at 5,000 x g for 10 minutes. 0.75 mL of the supernatant, in duplicate, were transferred to disposable polystyrene cuvettes and 0.25 mL of the chromagen solution and 0.50 mL of the sodium acetate buffer were added. Solutions were mixed by pipetting up and down and allowed to sit at room temperature for 1 hour to allow for color development. Absorbances were read at 535 nm against a water blank. A reagent blank was prepared using 0.75 mL water, 0.25 mL chromagen solution and 0.50 mL sodium acetate buffer and this amount was subtracted from the sample readings. Plasma iron concentration was determined by

comparing the adjusted sample readings to a standard curve generated using an Iron Atomic Absorption Standard Solution.

Liver iron concentration. Nonheme iron concentrations in collected tissues were determined by the colorimetric method described by Schricker and others (1983) as modified by Rhee & Ziprin (1987). This method was described in detail in Section 4.3. Briefly, finely chopped liver was mixed thoroughly with $NaNO_2$ and acidified. Tubes incubated in a water bath at 65°C for 20 hours and then cooled to room temperature. The acidic supernatant was mixed with a color reagent and centrifuged. The absorbance of the supernatant was read at 540 nm against the reagent blank. A sample blank, consisting of the acidic supernatant and the color blank reagent was read at 540 nm against the regent blank. Results were expressed as micrograms of nonheme iron per gram of liver tissue, wet weight.

TRFLP. Microbial DNA was extracted from adherent wash tubes using QIAamp DNA Stool Mini Kit (Qiagen; Valencia, CA, USA), according to manufacturer's instructions. Buffers and enzymes in the Stool Mini Kit lyse cells and stabilize nucleic acids prior to adsorbing DNA onto the QIAamp membrane. DNA is washed several times and then eluted into a centrifuge tube for PCR amplification. PCR amplification of the bacterial 16S rRNA gene was performed using the fluorescently labeled forward primer, 6-carboxyfluorescein-27f (6FAM-27f), and the reverse primer 1492r (Integrated DNA Technologies; Coralville, IA, USA). PCR reactions were performed using 10 ng of microbial DNA template in a 50 μL reaction mix containing 5 μL 10X *Ex Taq*™ Buffer (Mg^{2+} free), 4 μL $MgCl_2$ (25 mM), 5 μL *TaKaRa Ex Taq* ™ (5 units/μL), 4 μL dNTP Mixture (2.5 mM each) and 15 pmol of each primer. Reaction mix reagents, with the exception of the primers,

were sourced from TaKaRa Bio Inc (Shiga, Japan). PCR tubes were held at 95°C for 10 minutes prior to the start of thermocycling. The thermocycling conditions were 95°C for 30 seconds (opening of the DNA double helix producing single strand DNA), 53°C for 30 seconds (annealing of the primers to the single strand DNA), 72°C for 45 seconds (elongation of the strands by Taq); this cycle was repeated 29 more times and then the samples were held at 72°C for 10 minutes and then rapidly cooled to 4°C until removed from the PCR thermocycler.

PCR amplicons were loaded into wells of agarose gels (1% wt. agarose in 1x TAE [40 mM Tris, 20 mM acetic acid, and 1 mM EDTA], with 50 μL ethidium bromide added) and run at 125 V for 40 minutes in 1x TAE to check for purity and to ensure the correct band was amplified. Gels were viewed on a UV light box and cut for cleanup. PCR amplicons were purified using the Wizard SV Gel and PCR Cleanup (Promega), according to manufacturer's instructions. 500 ng of PCR product was digested with 20 u Bsh1236I in 10X Buffer R (supplied with enzyme; Fermentas; Glen Burnie, MD, USA) at 37°C for 14 hours. 500 ng of PCR product was also digested with 20 u of HhaI in 10 Buffer Tango™ (supplied with enzyme; Fermentas) at 37°C for 14 hours. Digestion products were cleaned-up using a Performa® DTR Ultra 96-well plate (BioEdge; Gaithersburg, MD, USA), which is a gel filtration plate that removes dNTPs, salts and primers from the DNA samples. HIDI formamide (Applied Biosystems) was added to all samples, along with 0.2 μL LIZ 600 bp size standard (Applied Bioststems). Samples were submitted to the Cornell University Life Sciences Core Laboratories Center (CLC) for fragment analysis. Relative abundances of phylotypes (peaks) were calculated similar to a method described by Jernberg *et al.* (2005).

Ribosomal database mining (Shyu *et al.* 2007) was used to determine putative phylotype identifications.

Analysis of mRNA in parotid glands. RNeasy Mini Kit (Qiagen) was used to extract RNA from the frozen parotid gland tissue according to manufacturer's instructions. Frozen tissues were lysed and homogenized prior to centrifugation to remove excess tissue. Lysates were adsorbed onto the RNeasy Mini spin column and washed several times prior to elution. Eluted RNA was frozen at -80°C until analysis. PCR amplification of the RNA was performed using Access RT-PCR kit (Promega), using primers PBII-F and PBII-R (Integrated Data Technologies), which correspond to the sequence of the human salivary proline-rich protein, PBRII. Ribosomal 18S was used to normalize the results. Thermocycling conditions were set to 2 min at 94°C, 1 min at 48°C, 2 min at 68°C for forty cycles, followed by 7 minutes at 68°C. PCR amplicons were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and then quantified using a Gel-Pro analyzer version 3.0 (Media Cybernetics LP, Silver Spring, MD, USA).

Proteomic analysis. Protein concentrations in the whole saliva samples were determined with the Bradford assay (Bradford 1976). The mean protein concentration of the whole saliva samples was 0.67 ± 0.29 μg/μL (range: 0.27-1.88 μg/uL). Whole saliva was centrifuged at 16,000 x g for 10 minutes to remove cellular debris. For analysis, sufficient saliva volume was pipetted such that 10 μg of protein from each whole saliva sample was placed into a centrifuge tube. Samples were evaporated in a speedvac until dry and then rehydrated with 10 μL of 2X SDS Sample Loading Buffer (125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, and 200 mM DTT). 10 μg of protein was loaded into each well of a

10-20% Novex® Tris-Glycine Gel (Life Technologies) and run at 125V for 1 hour 45 minutes. 1D gels were fixed in 40% (v/v) methanol/10% (v/v) acetic acid for 10 minutes prior to overnight staining with Colloidal Blue stain (Invitrogen) according to manufacturer's instructions. Gels were destained in distilled water.

To aid in the identification of PRPs, a second set of gels were run as described above and stained with 0.1% (wt/v) Coomassie Brilliant Blue R-250 (Invitrogen), for 3 hours in a 40% (v/v) ethanol/10% (v/v) acetic acid solution. With this procedure most proteins stain blue, but proline-rich proteins will stain pink (Beeley *et al.* 1991). These gels were destained in 10% (v/v) acetic acid for 24 hours at which point the pink colored proline rich protein bands could be distinguished from the others.

For analysis, whole saliva samples were pooled such that each saliva sample from a given treatment (Control, Black, Green) and given time point (day 0, 7, 14, 21, 28 or 35) contributed equivalent amounts of protein to the treatment pool.

Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA) using JMP 9.0.2 (SAS). When significant difference was detected by ANOVA, Tukey-Kramer test, which compares all means, was used to differentiate which samples were significantly different. Means were considered different at p-values less than 0.05.

Sample size was determined using JMP 9.0.2 (SAS) and data from our pilot pig study (2 animals per treatment group x 3 treatment groups for 10 days). It was calculated that a sample size of 33 animals (11 animals per treatment group) would be sufficient to detect differences in body weight between the three treatment groups using ANOVA with 90% power and a 5% significance level.

5.5 Results

5.5.1 Experiment 1

Body weight. There was no difference in body weight between any of the treatment groups at any given time point (p-values > 0.05; Figure 5-3 and Table 5-3).

Hemoglobin concentrations and hemoglobin repletion efficiency (HRE). There was no difference in hemoglobin concentrations, total body hemoglobin iron, daily feed intake or HRE values between the treatments (Table 5-4). It was observed that all treatments experienced a dip in hemoglobin concentration between Week 0 and Week 1 but this finding was not statistically significant.

Serum iron concentrations. Serum iron concentrations (Table 5-4) were not significantly different between treatments, at a given time point. However, there were significant decreases in the mean serum iron concentrations of the Control group (p=0.038) and the Green Tea group (p=0.024) at Week 1. Similarly a small dip in serum iron was observed at Week 1 in the Black Tea group, but this was not significant (p=0.1686).

5.5.2 Experiment 2

Body weight. There was no difference in growth between any of the treatment groups at any given time point (p-values > 0.05; Figure 5-4 and Table 5-5).

Hemoglobin concentrations. Hemoglobin concentrations were not significantly different between treatments at any time point (Table 5-6). There was a small but not statistically significant drop observed in hemoglobin concentration at Week 2.

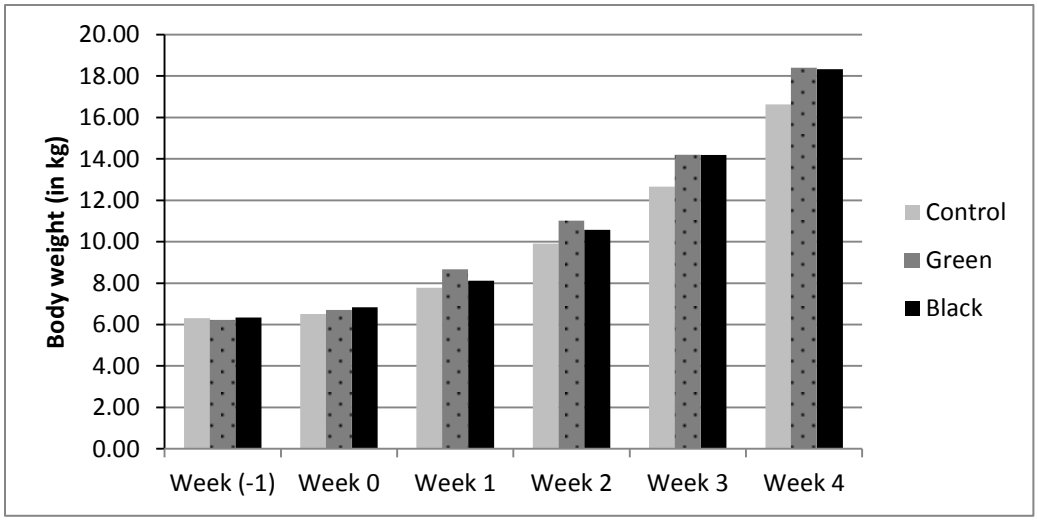


Figure 5-3. Weekly mean body weights (Experiment 1). See Table 5-3 for standard deviations and the results of statistical analysis.

Table 5-3. Body weight comparisons among treatment groups (Experiment 1). Means and standard deviations in parenthesis, n=9 animals per treatment group, where indicated by (*), n=8 animals per treatment group.

	Mean Body Weight (in kg)					
Group	Week (-1)	Week 0	Week 1	Week 2	Week 3	Week 4
Control	6.31	6.51	7.78	9.91	12.65	16.63
	(0.78)	(1.12)	(1.77)	(3.29)	(5.19)	(6.94)
Green Tea	6.24	6.71	8.67	11.02	14.21	18.41
	(0.85)	(1.34)	(1.79)	(2.72)	(3.71)	(4.56)
Black Tea*	6.34	6.83	8.11	10.58	14.18	18.33
	(0.80)	(1.27)	(2.13)	(3.29)	(4.04)	(4.91)

Table 5-4. Hemoglobin concentrations, total body hemoglobin iron content, daily feed intake, hemoglobin repletion efficiency and serum iron concentration comparisons between treatments (Experiment 1). Means ± standard deviations. Values in the same row with different letters are considered significantly different.

	Week 0	**Week 1**	**Week 2**	**Week 3**	**Week 4**
			Hemoglobin concentration (g/dL)		
Control	10.65 ± 1.20	9.38 ± 1.24	9.75 ± 1.65	9.59 ± 2.12	9.49 ± 3.19
Green Tea	10.80 ± 2.56	10.48 ± 1.88	10.90 ± 2.21	10.91 ± 2.14	10.83 ± 2.62
Black Tea	10.68 ± 1.36	9.71 ± 1.60	10.29 ± 1.92	10.62 ± 2.58	11.00 ± 2.13
			Total body Hb Fe content (in mg)		
Control	154 ± 28	166 ± 56	226 ± 112	290 ± 178	392 ± 265
Green Tea	164 ± 52	209 ± 72	281 ± 114	362 ± 143	469 ± 193
Black Tea	165 ± 44	185 ± 73	262 ± 120	360 ± 166	469 ± 182
			Daily feed intake (in g)		
Control		376 ± 137	548 ± 269	665 ± 364	865 ± 442
Green Tea		436 ± 137	603 ± 198	728 ± 230	825 ± 243
Black Tea		404 ± 160	652 ± 176	802 ± 190	908 ± 262
			Hemoglobin repletion efficiency (in %)		
Control		14.48 ± 17.84	16.31 ± 17.40	17.86 ± 16.25	20.62 ± 18.94
Green Tea		27.10 ± 21.32	28.89 ± 14.33	29.50 ± 10.05	31.42 ± 11.41
Black Tea		18.19 ± 21.13	22.38 ± 15.55	27.17 ± 13.68	29.58 ± 11.33
			Serum iron concentration (in μg/ml)		
Control	$0.21 \pm .019^{AB}$	0.07 ± 0.08^{B}	0.29 ± 0.13^{A}	0.33 ± 0.25^{AB}	0.23 ± 0.17^{AB}
Green Tea	0.62 ± 0.60	0.12 ± 0.16	0.23 ± 0.15	0.25 ± 0.19	0.33 ± 0.32
Black Tea	0.31 ± 0.49^{A}	0.01 ± 0.02^{B}	0.21 ± 0.12^{AB}	0.20 ± 0.08^{AB}	0.23 ± 0.12^{AB}

Serum iron concentrations. Serum iron concentrations were not significantly different between treatments at any time point, nor different within a treatment between time points (Table 5-7).

Liver iron stores. Liver nonheme iron was lowest in the pigs fed green tea and highest in the control pigs (p=0.0014; Table 5-8).

Intestinal microbiota. In order to characterize the differences in the microbial community between treatments, each individual peak area was converted to a percentage abundance value by dividing the raw peak (phylotype) area by the sum of all peak areas on the given spectrum (Jernberg *et al.* 2005). Similar to Patterson *et al.* (2009), individual phylotypes were ranked according to abundance and the 30 most abundant phylotypes were considered dominant. Fragments with putative identification that were identified in our samples are presented in Table 5-9 (Cecum) and Table 5-10 (Colon).

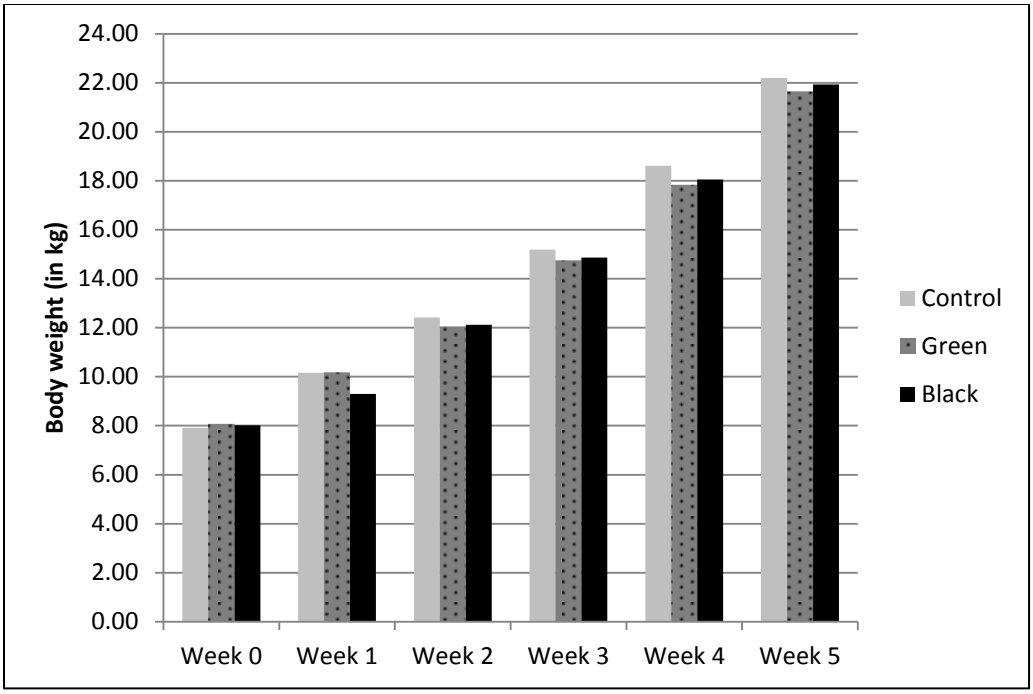


Figure 5-4. Weekly mean body weights (Experiment 2). See Table 5-5 for standard deviations and the results of statistical analysis.

Table 5-5. Body weight comparisons among treatment groups (Experiment 2). Means and standard deviations in parenthesis, n=10 animals per treatment group.

	Body Weight (in kg)					
Group	**Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
Control	7.91	10.17	12.42	15.20	18.62	22.20
	(0.99)	(3.01)	(4.85)	(6.71)	(9.10)	(10.41)
Green	8.07	10.18	12.04	14.74	17.84	21.65
	(0.78)	(1.93)	(3.36)	(5.23)	(6.82)	(8.66)
Black	8.02	9.31	12.12	14.87	18.06	21.94
	(1.03)	(2.09)	(3.58)	(4.66)	(6.19)	(7.83)

Table 5-6. Hemoglobin concentration and total body hemoglobin iron content comparisons between treatments and time points (Experiment 2). Means ± standard deviations, n=10 animals per treatment group.

	Week 0	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
	Hemoglobin concentration (in g/dL)					
Control	8.56 ± 2.14	7.83 ± 1.54	7.70 ± 1.59	7.90 ± 1.86	7.85 ± 1.72	8.70 ±1.73
Green	7.95 ± 1.93	7.16 ± 1.76	7.05 ± 1.57	7.41 ± 1.55	7.28 ± 2.10	8.34 ± 2.40
Black	8.28 ± 1.49	7.78 ± 1.41	7.41 ± 1.38	7.37 ± 1.35	7.54 ± 1.35	8.89 ± 1.85
	Total body Hb Fe content (in mg)					
Control	156 ± 56	186 ± 87	228 ± 130	292 ± 186	355 ± 236	465 ± 289
Green	145 ± 44	168 ± 71	198 ± 96	257 ± 139	316 ± 199	441 ± 283
Black	150 ± 41	166 ± 58	208 ± 88	257 ± 114	320 ± 152	463 ± 230

Table 5-7. Serum iron concentrations (Experiment 2). Means ± standard deviations, n=10 animals per treatment group.

	Serum iron concentration (in µg/mL)				
Group	**Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Control	0.35 ± 0.23	0.39 ± 0.28	0.50 ± 0.29	0.39 ± 0.20	0.39 ± 0.21
Green	0.29 ± 0.13	0.37 ± 0.25	0.40 ± 0.11	0.36 ± 0.16	0.39 ± 0.24
Black	0.42 ± 0.23	0.34 ± 0.13	0.41 ± 0.16	0.41 ± 0.15	0.40 ± 0.21

Table 5-8. Nonheme liver iron concentration, in µg/g liver, wet weight (Experiment 2). Means ± standard deviations, *n*=5 animals per treatment group.

Treatment	**Liver Iron Content (in µg/g liver)**
Day 0	4.48 ± 0.39 ^{AB}
Control	5.05 ± 0.63 ^{A}
Black	4.52 ± 0.85 ^{AB}
Green	4.01 ± 0.53 ^{B}

In the cecum, the piglets fed black tea showed at 68% decrease in *Clostridium* spp. (phylotype 60) compared to the control, while piglets fed green tea showed similar phylotype 60 (P60) abundance as the control. The piglets fed green tea also showed similar abundances of P224 and P229, both corresponding to *Clostridium* spp, while these phylotypes were not identified in the piglets fed black tea. The abundance of P61 (*Lactobacillus* spp.) was increased 79% in the piglets fed green tea but decreased by 40% in the piglets fed black tea, as compared to the control. An increase of P103 (*Lactobacillus* spp.) was observed in the piglets fed black tea, as compared to the control, but not in the piglets fed green tea. *Bifidobacterium* spp (phylotype 227) was identified only in the pigs fed black tea. In general, Clostridia (P60, P224, P229) was reduced in the piglets fed black tea ($p < 0.05$), but not significantly in piglets fed green tea, as compared to the control. Lactobacilli appeared to be similar across all groups.

In the colon, increases of P103 (*Lactobacillus* spp.) 208% and 177% were observed in piglets fed green or black tea, respectively. *Clostridia* (P60, P224, P229 and P131) were similar between piglets fed green tea and the control piglets, although a 48% reduction was observed for piglets fed black tea. P207 (*Bifidobacterium* spp.) was observed only in the control piglets, while P227 (*Bifidobacterium* spp.) was observed only in the piglets fed green tea.

Table 5-9. Phylotypes with putative identifications detected on TRFLP profiles of the cecum of piglets. Most abundant species are indicated.

Phylotype	*Genus*	*Cecum*			
		Day Zero	Control	Green	Black
60	Clostridium spp.				
61	Lactobacillus spp.				
103	Lactobacillus spp.				
105	Mycobacterium/Flavobacter spp.				
107	Flexibacter/Flavobacter/Cytophaga spp.				
111	Flexibacter/Flavobacter/Cytophaga spp.				
113	Streptococcus/Lactococcus spp.				
117	Prevotella spp.				
119	Prevotella spp.				
122	Streptococcus/Eubacterium spp.				
124	Eubacterium spp.				
131	Clostridium/Eubacterium spp.				
138	Eubacterium spp.				
140	Eubacterium spp.			■	
203	Clostridium spp.				
207	Bifidobacterium spp.				
221	Streptococcus/Prevotella spp.		■		
223	Clostridium/Mycobacterium spp.				
224	Clostridium spp.	■	■	■	
226	Clostridium spp.				■
227	Bifidobacterium spp.				
229	Clostridium spp.		■		
240	Lactobacillus/Clostridium spp.	■			
241	Lactobacillus/Clostridium spp.		■		■
249	Enterococcus/Lactobacillus spp.				
274	Campylobacter spp.				■
275	Campylobacter spp.				■
393	Clostridium/Campylobacter spp.				■
394	Enterobacteriaceae/Campylobacter spp.			■	
401	Bacillus/Paenibacillus spp.				
534	Lactobacillus/Paenibacillus spp.				■

Table 5-10. Phylotypes with putative identifications detected on TRFLP profiles of the colon of piglets. Most abundant species are indicated.

Phylotypes	Genus	Colon			
		Day Zero	Control	Green	Black
60	Clostridium spp.				
61	Lactobacillus spp.				
103	Lactobacillus spp.				
105	Mycobacterium/Flavobacter spp.				
107	Flexibacter/Flavobacter/Cytophaga spp.				
111	Flexibacter/Flavobacter/Cytophaga spp.				
117	Prevotella spp.				
119	Prevotella spp.				
121	Streptococcus/Prevotella spp.				
125	Eubacterium spp.				
131	Clostridium/Eubacterium spp.				
138	Eubacterium spp.				
140	Eubacterium spp.				
203	Clostridium spp.		■		
207	Bifidobacterium spp.				
221	Streptococcus/Prevotella spp.				
223	Clostridium/Mycobacterium spp.	■	■		
224	Clostridium spp.				
226	Clostridium spp.				
229	Clostridium spp.				
240	Lactobacillus/Clostridium spp.				
241	Lactobacillus/Clostridium spp.				
274	Campylobacter spp.			■	
275	Campylobacter spp.		■		
380	Bacillus spp.				
394	Enterobacteriaceae/Campylobacter spp.			■	
401	Bacillus/Paenibacillus spp.		■		
408	Lactobacillus/Paenibacillus/Clostridium spp.				
491	Clostridium spp.			■	
533	Lactobacillus/Paenibacillus spp.	■			

Analysis of mRNA in parotid glands. Parotid gland mRNA abundances of the proline rich protein are presented in Table 5-11. There was no difference in mRNA abundances between the three groups or the Day Zero control animals (p=0.836).

Table 5-11. Relative abundances of proline-rich protein, PRBII, expressed relative to the expression of 18S rRNA (arbitrary units, AU). n=5 (Control, Green Tea, Black Tea) or n=2 (Day Zero).

Group	PRB-II Relative Abundance (in AU)
Day Zero	1.0502 ± 0.0008
Control	1.0504 ± 0.0009
Green Tea	1.0500 ± 0.0006
Black Tea	1.0508 ± 0.0021

Proteomic Analysis. 1D SDS-PAGE gels are presented in Figure 5-5 (stained with Colloidal blue) and Figure 5-6 (stained with Coomassie R-250). Presumed PRPs are indicated by pink color (Beeley *et al.* 1991) and arrows in Figure 5-6. Similar to other published results (Gutierrez *et al.* 2011), we found obvious variation in whole saliva protein composition, not only between samples from different groups but also within the same group. However, comparisons of pooled group saliva from day 0, day 28 and day 35, revealed some general trends in the saliva proteome. Figure 5-5 demonstrates than an increase in proteins around 115 kDa was observed in the tea groups on Day 35. The pink color of this band in Figure 5-6, indicates that these proteins may be PRPs because the Coomassie stain selectively stains PRPs pink. There did not appear to be a noticeable trend in the other bands containing presumed PRPs (~25 and 7 kDa).

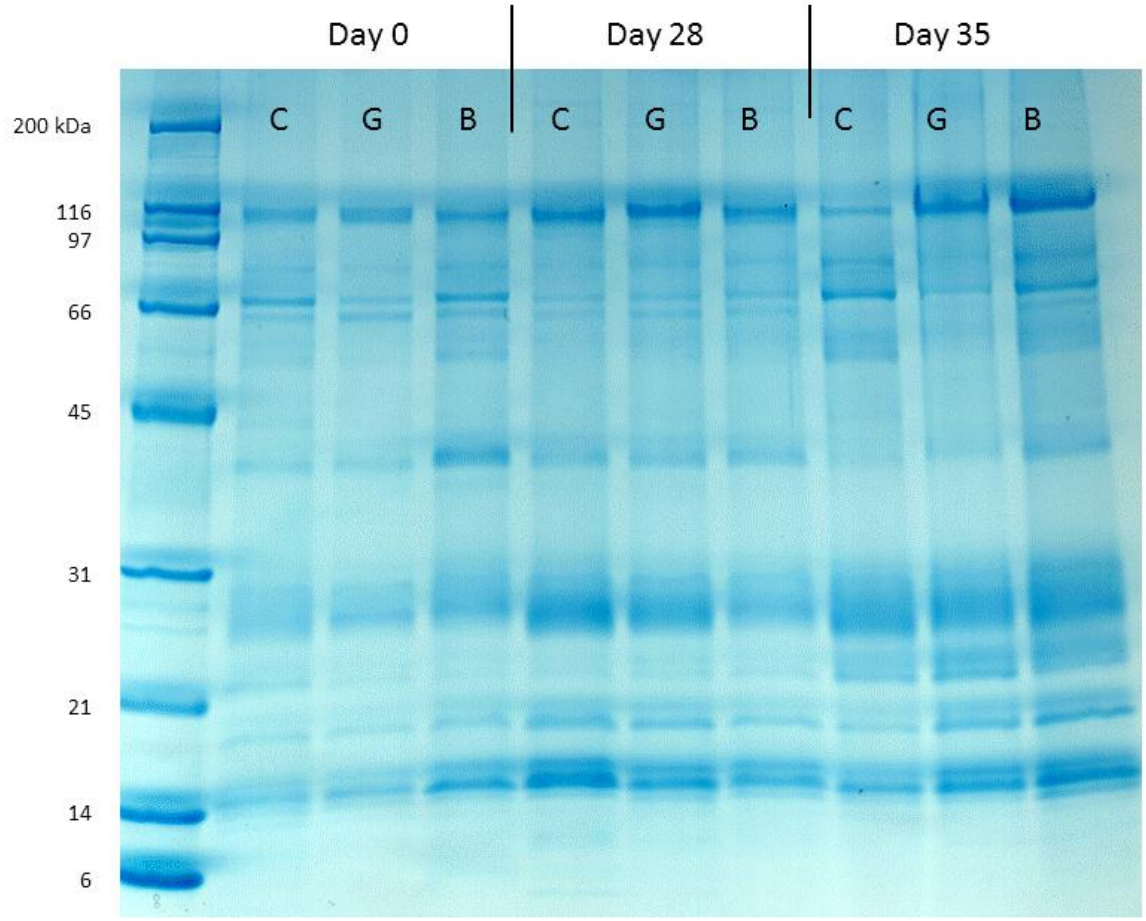


Figure 5-5. 1D SDS-PAGE on Tris-glycine gels (10-20%). Proteins (10 ug, according to protein determination) were reduced and applied per lane. After separation, bands were stained with Colloidal blue. From left to right, lanes correspond to the molecular weight marker, followed by group pools of Day 0 Control, Day 0 Green, Day 0 Black, Day 28 Control, Day 28 Green, Day 28 Black, Day 35 Control, Day 35 Green and Day 35 Black.

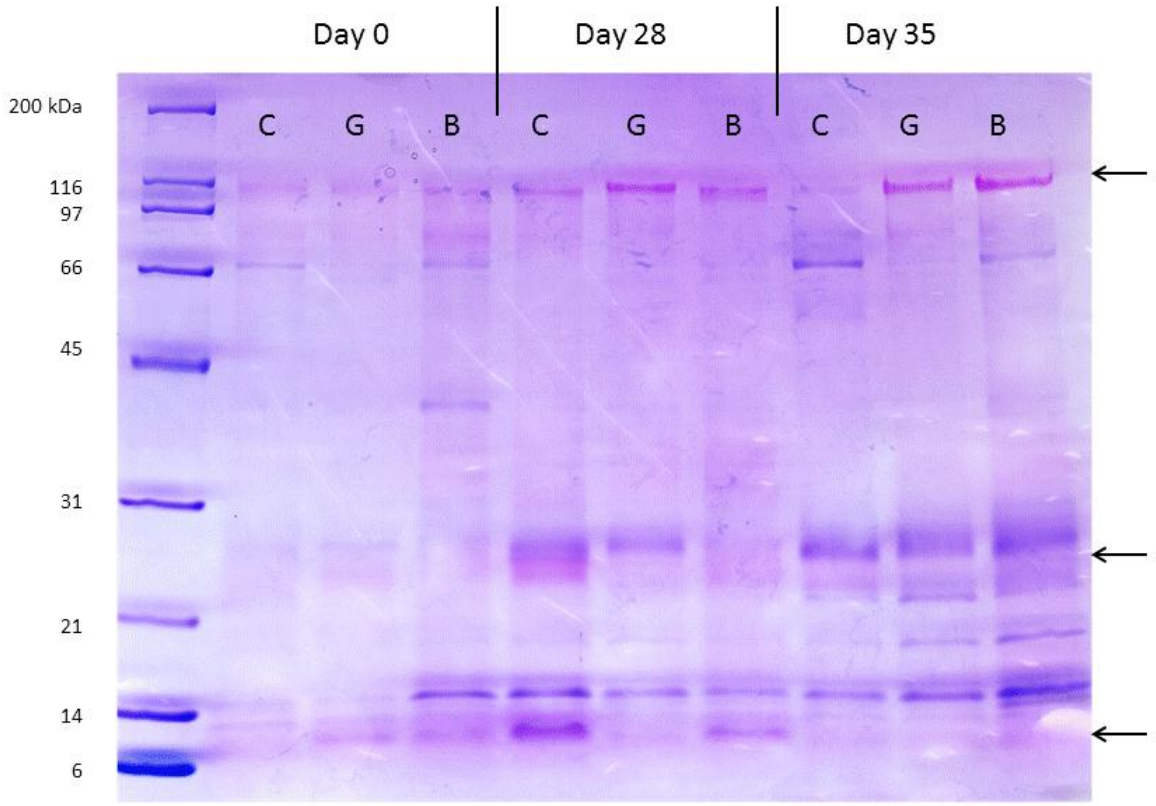


Figure 5-6. 1D SDS-PAGE on Tris-glycine gels (10-20%). Proteins (10 ug, according to protein determination) were reduced and applied per lane. After separation, bands were stained with Coomassie R-250. From left to right, lanes correspond to the molecular weight marker, followed by group pools of Day 0 Control, Day 0 Green, Day 0 Black, Day 28 Control, Day 28 Green, Day 28 Black, Day 35 Control, Day 35 Green and Day 35 Black.

5.6 Discussion

In experiment 1, 2 green tea pigs, 2 black tea pigs and 2 control pigs were removed from the study due to loss of weight and/or refusal to eat. Animals removed from the iron-deficient diet resumed normal behavior within one week on a commercial diet, although they were consistently smaller than their littermates that were not used on this study. An additional black tea pig was removed and euthanized due to refusal to eat, even after removal from the study.

In experiment 1, it was observed that there were large variations in body weight within groups. Some control piglets grew less than 5 kg in 4 weeks while others gained over 15 kg. A similar range was observed within the black tea groups and the green tea groups. However, despite the range, the average weights of the control, green tea and black tea groups (16.63± 6.94, 18.41 ± 4.56 and 18.33 ± 4.91, respectively), were lower than the average weight of pigs 8-10 weeks of age, reportedly 21-30 kg (Carr 1998). The animals in experiment 1 were not given antibiotics and it was theorized that the poor gastrointestinal health of some of the pigs, as evidenced by a high diarrhea prevalence, could have inhibited their ability of grow, regardless of the basal or tea diet. It is well documented that the environmental and dietary changes experienced by weanling piglets can cause stress resulting in intestinal disorders (Zanchi *et al.* 2008). For this reason, piglets of this age are usually given antibiotics to prevent the overgrowth of harmful bacteria, which can result in increased diarrhea and decreased nutrient absorption (Fairbrother *et al.* 2005). If the observed diarrhea was evidence of an unfavorable microbial balance, then it was hypothesized that the addition of antibiotics to the piglet feed, would reduce the effects of

diarrhea, possibly allowing for the differentiation between piglets fed the control diet and those fed tea. Therefore, at the advice of the swine farm manager, 0.5% (by weight) tylosin (an antibiotic) was added to the piglet diets in experiment 2.

In experiment 2, piglets were housed in group pens because the weather conditions at the time of the study disallowed the use of the Block Barn, as the barn temperatures would have been too low. As a result, pigs were housed in 3 group pens (control, green, black) for two weeks before transport to 6 group pens (2 each control, green, and black) for the remaining 3 weeks of the study. During the 5-week study, 5 piglets (2 control, 2 green tea and 2 black tea) were removed due to loss of weight over a two week period and 1 black tea piglet was removed due to extremely low growth after three weeks on the study.

Despite the addition of an antibiotic, variation within groups was similar to what was observed in the first experiment. There was no significant difference in the weight gain between the groups within experiment 1 (p=0.830) or experiment 2 (p=0.477) , nor was there any significant difference in piglet weight gain between piglets in experiment 1 and experiment 2 at 4 weeks (p=0.110). It should be mentioned that incidences of diarrhea were lower in the antibiotic fed pigs, possibly indicating better gastrointestinal health (Bruins *et al.* 2011). It was observed that the pigs fed green tea had liver nonheme iron stores that were lower than the stores of the control, but not lower than the Day 0 pigs or the pigs fed black tea. It is possible that green tea inhibited iron absorption slightly, but not significantly, as compared to the control.

Taken together these results indicate that green or black tea ingestion does not significantly affect iron absorption in anemic pigs, as measured by hemoglobin repletion

efficiency and weight gain. This finding is similar to a report from Sarker *et al.* (2010), which demonstrated that pigs with 0.5%-2% green tea powder added to their diets did not have body weights significantly lower than the control, after a six-week study. It should be noted that there were substantial differences between their study and the one described here. In their 6-week study, finishing pigs (initial weights ~70 kg) fed nutritionally complete diets were used, whereas in this study, iron-deficient weanling piglets were used and fed an iron-poor diet. A fair criticism is that the iron-replete status and adequate iron intake of the finishing pigs on the 6-week study might have hid any inhibitory effects of tea consumption. In the study presented here, the large variation in weight gain, serum iron and HRE could have also hidden the inhibitory effects of tea polyphenols.

However, in defense of this study, the iron deficient piglet model has been shown to be an effective model for estimating the iron absorption in humans (Tako *et al.* 2009). Piglets have been shown to be a better model than the more commonly used rat, because although they are larger and more difficult to work with, pigs have gastrointestinal tracts that are much more similar to humans than that of a rat to a human (Greger 1992). Furthermore, iron deficiency can easily be induced in piglets by decreasing the amount of iron injected at birth (Zimmermann 1980), owing to their fast growth and high iron demand to support such growth (Patterson *et al.* 2008). As a result, researchers can adjust the iron status of their piglets, making them iron-deficient and therefore possibly more sensitive to iron inhibitors.

In this study, similar to Tako *et al.* (2009), hemoglobin repletion efficiency (HRE) was used to assess iron bioavailability by monitoring the ability of the piglet to utilize ingested

iron to increase hemoglobin content to a normal level. It was assumed that if piglets were mildly anemic, most of their ingested iron would be used for hemoglobin synthesis not stored in ferritin and that body hemoglobin content could be used to monitor bioavailability over time. The question posed by this study is: at what level are the pigs too anemic and unable to recover?

A graph of initial hemoglobin versus weight gain at 4 weeks was plotted for all pigs in both experiment 1 and experiment 2 (Figure 5-7). With a few exceptions, pigs with initial hemoglobin values of over 8 g/dL gained more weight than pigs with initial hemoglobin values less than 8 g/dL. This follows the published values that a minimum hemoglobin concentration of 9 g/dL is needed for good growth in pigs and levels of 8 g/dL likely indicates anemia (Zimmermann 1980). Further indication that the study pigs were anemic was the observation that most of the study pigs had very low serum iron (<1 μg/mL). When serum iron was plotted against hemoglobin concentrations (Figure 5-8), it appeared that even pigs with higher hemoglobin concentration had very low serum iron. It is important to note that while some of the values in Figure 5-8 appear to indicate that some of the pigs had serum iron concentrations of 0 μg/mL, this is more an indication that our test is not sensitive enough to detect very, very low serum iron concentrations (<0.01 μg/mL). However, Figure 5-8 does indicate that some of our animals had very low serum iron concentrations, a further indication of iron deficiency. If some of the study animals were severely anemic, while others were mildly anemic to just iron deficient, there would be significant differences in their ability to grow and this could explain why there was such variation, even within groups.

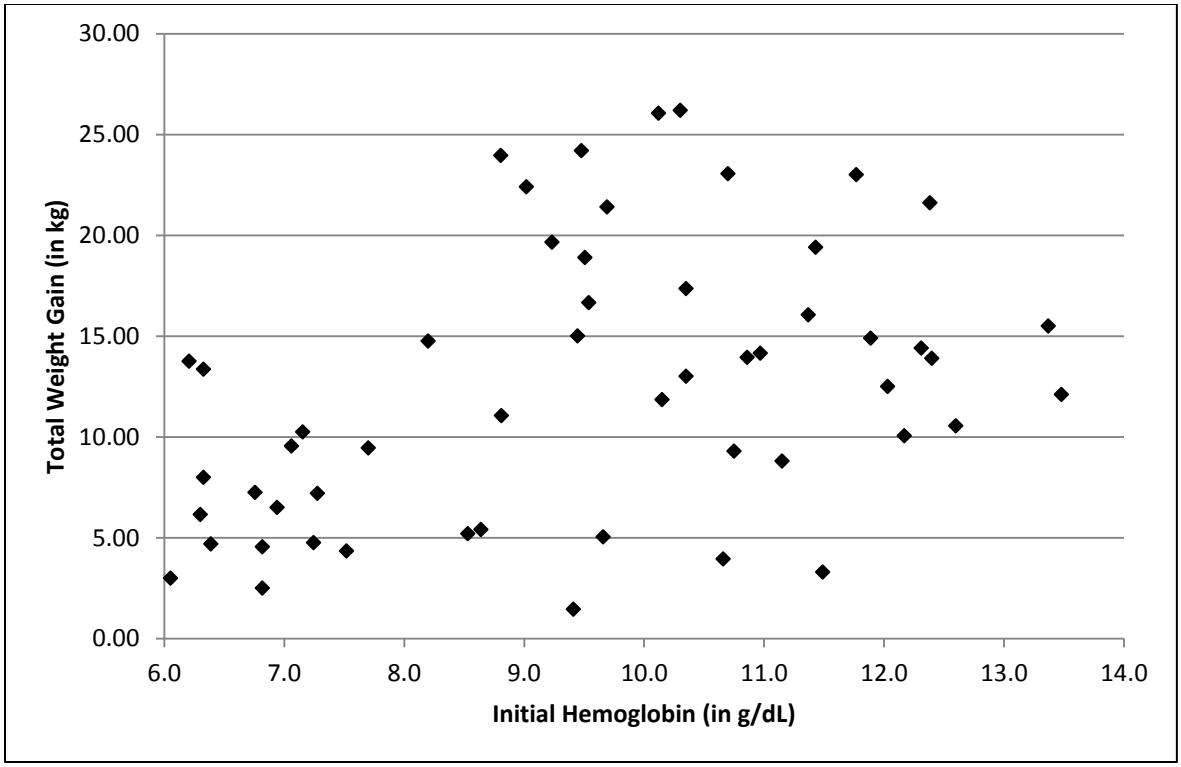


Figure 5-7. Initial hemoglobin concentration versus weight gain after 4 weeks (experiment 1 and experiment 2 data combined). Positive association was observed, p=0.035, R^2=0.254.

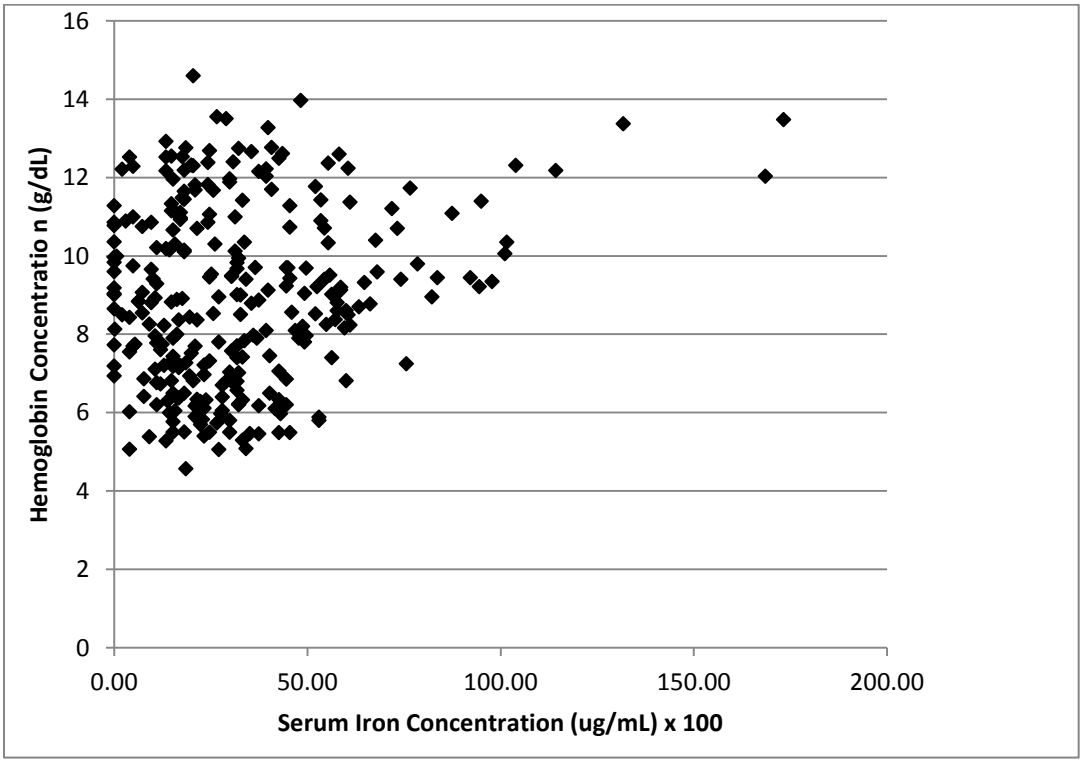


Figure 5-8. Hemoglobin concentration versus serum iron concentration over 4 weeks (experiment 1 and experiment 2 data combined).

The fact that some of the pigs grew equally well or equally poor in the tea treatments versus the control, suggests that tea did not have a major effect on the growth of the piglets. This is not the only study to observe no effect of dietary polyphenols on iron absorption in a piglet model. In a 5-week study of the inhibitory effect of red bean polyphenols, Tan *et al.* (2008) report that there was no effect on weight gain or HRE after feeding pigs red beans in the diet, compared to white beans. Similarly, Jansman *et al.* (1993) reported no difference in body weight between piglets put on a high tannin diet versus a control tannin-free diet. In a study utilizing acorn shred with a high tannin content, Cappai *et al.* (2010) found that while piglets fed a 70:30 acorn shred:complete diet did not have weight gain different than animals fed the complete diet only, but they had larger parotid glands and higher proline content in the parotid gland. The authors suggested that piglets have the ability to increase their proline rich protein expression, similar to how rats can increase their PRP expression in the presence of tannins (Mehansho *et al.* 1983). While Jansman *et al.* (1993) did not find an increase in the parotid gland size in pigs fed a high tannin diet, Tan *et al.* (2008) did report that PRP mRNA concentration was twice as high in the parotid glands of iron-deficient piglets fed red beans versus white beans. The authors proposed that while hemoglobin concentration, HRE and weight were not different, the increased PRP mRNA indicated that increased PRP expression protected piglets from the inhibitory effects of red bean polyphenols.

It is interesting that in the study presented here PRP mRNA concentration was not different between the groups. However, 1D SDS-PAGE of saliva seemed to indicate that there was an increase in proteins rich in proline within the band of proteins at 115 kDa in the tea groups. A possible reason for the discrepancy is that only the 5 largest pigs from

each treatment were chosen to be sacrificed, whereas for SDS-PAGE all animals within a treatment at a given time point were pooled. The five largest pigs from each treatment were chosen to be sacrificed because it was reasoned that these animals were the best adapted to their treatment. It is theoretically possible that the five largest piglets had sufficient baseline PRP expression that they did not need to increase expression to get over the effects of tea feeding. However it is more likely that the proline-rich protein(s) corresponding to the band at 115 kDa is not coded for by the PBRII gene. Perhaps the amount of tea in the diet was insufficient to cause increased expression of the PBRII gene. However, the amount of tea used in this study was relevant to the amounts consumed by humans. Early in the study, smaller pigs consumed around 200 grams of feed daily, while at the end of the study the biggest pigs were consuming almost 1000 grams daily. Given that the average cup of water-extracted tea is 0.3% tea solids (Quinlan et al. 1997), the pigs consumed the equivalent of 650 ml to 3.3 liters of tea per day.

The final aspect of this study was to observe the effects of tea feeding on the gut microbial community. *In vitro*, tea polyphenols and extracts have been shown to have antibacterial properties against *Staphylococcus aureus*, some Clostridia, including *Clostridium perfringens*, and other pathogenic bacteria, while enhancing some Bifidobacteria (Hara *et al.* 1995; Bruins *et al.* 2011). In the piglet model, Hara *et al.* (1995) found that 2% tea polyphenols in the feed increased Lactobacilli and decreased total bacterial counts. In a more recent study, Zanchi *et al.* (2008) found that adding whole tea plant extract, a byproduct of tea manufacturing, decreased Clostridia and Enterococci. In the study

presented here, Clostridia were reduced in the piglets fed black tea, while increases in Lactobacilli were observed in both tea groups, as compared to the control.

While these observations indicate support to published findings that black tea may favor beneficial bacteria while inhibiting the growth or colonization of harmful populations, it is important to consider that these animals (experiment 2) received antibiotics in their feed. After the high instance of diarrhea in experiment 1, which indicated gastrointestinal issues, it was decided that it was best to administer antibiotics to the animals in experiment 2. The objective of these experiments was to investigate the effects of long term green or black tea consumption on iron absorption and in order to preserve the integrity of the experiment, it was believed at the time, that it was necessary to add antibiotics to the animal feed. Typically in experiments where the objective is to study the microbial populations in the intestines, antibiotics are used as a treatment (versus a control) or not at all (Blachier *et al.* 2007; Zanchi *et al.* 2008; Patterson *et al.* 2009). Regardless, the observation of increased Lactobacilli and decreased Clostridia in the tea groups, as compared to the control, demonstrates that the animals fed tea had better gut microbial health (Patterson *et al.* 2009), indicating that tea consumption positively affects the gut microbial community by either preventing the growth of undesirable bacteria or promoting the growth of beneficial bacteria.

5.7 Conclusions

Neither green nor black tea affected the weight gain, hemoglobin concentration or hemoglobin repletion efficiency in iron-deficient weanling piglets. PRP mRNA expression was also not different among the groups but 1D SDS-PAGE revealed an increase of

proteins rich in proline in the tea groups, when compared to the control. It is possible that an increase of PRP expression could be why tea consumption had no effect on iron absorption measures. It is also possible that some piglets were too severely anemic to be able to recover from their extremely iron deficient state and therefore any treatment effect was masked. The effect of tea consumption in a less iron-deficient piglet model may be more useful in investigating the effect of long term tea consumption on the piglet.

CONCLUDING REMARKS

Fortified, extruded rice has the potential to be a powerful vehicle for micronutrient delivery in the developing world, as many populations in those countries eat mostly plant-based diets and consume rice regularly. Although, electrolytic iron has a bioavailability lower than that of a more soluble iron fortificant, like ferrous sulfate, it has excellent potential for use in extruded rice kernels because it did not cause unwanted color changes within the extruded kernels. The addition of zinc, vitamin A and ascorbic acid to the electrolytic iron-fortified kernels did not cause any color changes, but did increase iron dialyzability from the extruded kernel. These results suggest that the formula containing electrolytic iron, zinc oxide, retinyl palmitate and ascorbic acid would be beneficial in alleviating micronutrient deficiencies within a rice eating population.

The addition of Vitamin A, before or after cooking did not affect the iron dialyzability from corn porridge, when compared to the iron fortified control. It was hypothesized that perhaps the addition point of vitamin A could explain why there are contradictory reports of the effect of vitamin A on iron absorption. A Venezuelan research group reported that vitamin A significantly increases iron from a meal, while another research group found no absorption increase whether the study population was young, healthy personals or vitamin A deficient ones. However, the results of the *in vitro* digestion indicated that there was no effect on iron dialyzability, suggesting low or no effect on bioavailability *in vivo*. To better investigate the discrepancy between the two research groups' findings, more research needs to be performed using human subjects. Iron absorption results obtained using non-human models, whether an animal model or *in vitro*

techniques, can mimic certain processes within humans, but cannot replace the use of human studies. In the case of vitamin A, to understand the discrepancies between the findings, this point must be further investigated using human subjects.

Animal studies were conducted to test the hypothesis that proline rich proteins could counteract the effects of tea polyphenols on iron absorption. In rats, tea consumption was not observed to cause any decreases in iron absorption, liver iron stores or weight gain, however the addition of tea to the diet stimulated significant changes in the expression of proteins in whole saliva. It was observed that there was a marked increase in PRP expression in whole saliva indicating that the increased secretion of PRPs may help the rat adapt to the inhibitory effects of tea polyphenols.

In pigs, neither green nor black tea consumption caused any decrease in weight gain, hemoglobin concentration or hemoglobin repletion efficiency. However, there was concern that some of the piglets may have been too anemic and that the variation within groups hid any possible treatment effect. As such, suggested future research is to investigate the effects of tea feeding on pigs that are less anemic. Based on observations from our study, a hemoglobin concentration of not less than 9 g/dL would be preferred. As for PRPs in pigs, tea feeding caused an apparent increase in proteins rich in proline but did not significantly increase the concentration of PRP mRNA. However, other studies in pigs have shown that pigs increase PRP expression when fed diets high in inhibitory polyphenols. Still, more research is needed in this area studying the degree to which pigs are able to increase PRP secretion in response to dietary polyphenols.

Additionally, humans naturally secrete high amounts of PRPs in saliva, and researchers need to investigate whether humans have the ability to increase their PRP secretion in response to changes in the diet. If humans have the ability to modify the salivary proteome in response to dietary changes, that would provide valuable insight into why tea affects iron absorption in single-meal studies but there is no association between tea consumption and iron status in populations. However, if humans do not have the ability to modify the salivary proteome in response to tea drinking, then there is some other adaptation mechanism that must be uncovered.

A final observation from this thesis is that black tea appeared to have a beneficial effect on the gut microbial community by favoring Lactobacilli and inhibiting Clostridia. It appears that tea affects the gut microbiota by inhibiting the growth of Clostridia and other pathogenic bacteria, which allows other more beneficial bacteria, such as Lactobacilli to grow. However, more research is needed in this area. The use of piglets that are not iron deficient, but that are also not given antibiotics, would be a good proxy for human subjects. If tea is able to favorably affect the gut microbial community in piglets, this could justify performing research in this area with human subjects. If tea is able to favorably affect microbial populations in humans, those findings would be very exciting contributions to the present body of work surrounding human health. Currently, little research has been performed in this area and so there is much room for investigating the effect of tea on gastrointestinal health.

Taken together, the *in vivo* studies presented in this thesis demonstrate that tea drinking does not inhibit iron absorption and that during long term consumption, the body

adapts to tea in the diet. These findings, along with human epidemiological studies suggest that humans may have a mechanism for adapting to dietary polyphenols. More research about human adaptation to dietary polyphenols is key to understanding if increased PRP—or other salivary protein—secretion in humans protects the body against habitual polyphenol ingestion. It would be particularly beneficial to study the salivary proteome in humans before and after long term tea drinking. It would be important to use non-tea drinkers in this research and to monitor any changes in their saliva from before the tea regimen and after. Research in humans is necessary to understand why single-meal studies have shown that tea decreases iron absorption, but in studies of populations, iron status is not associated with tea drinking.

APPENDIX

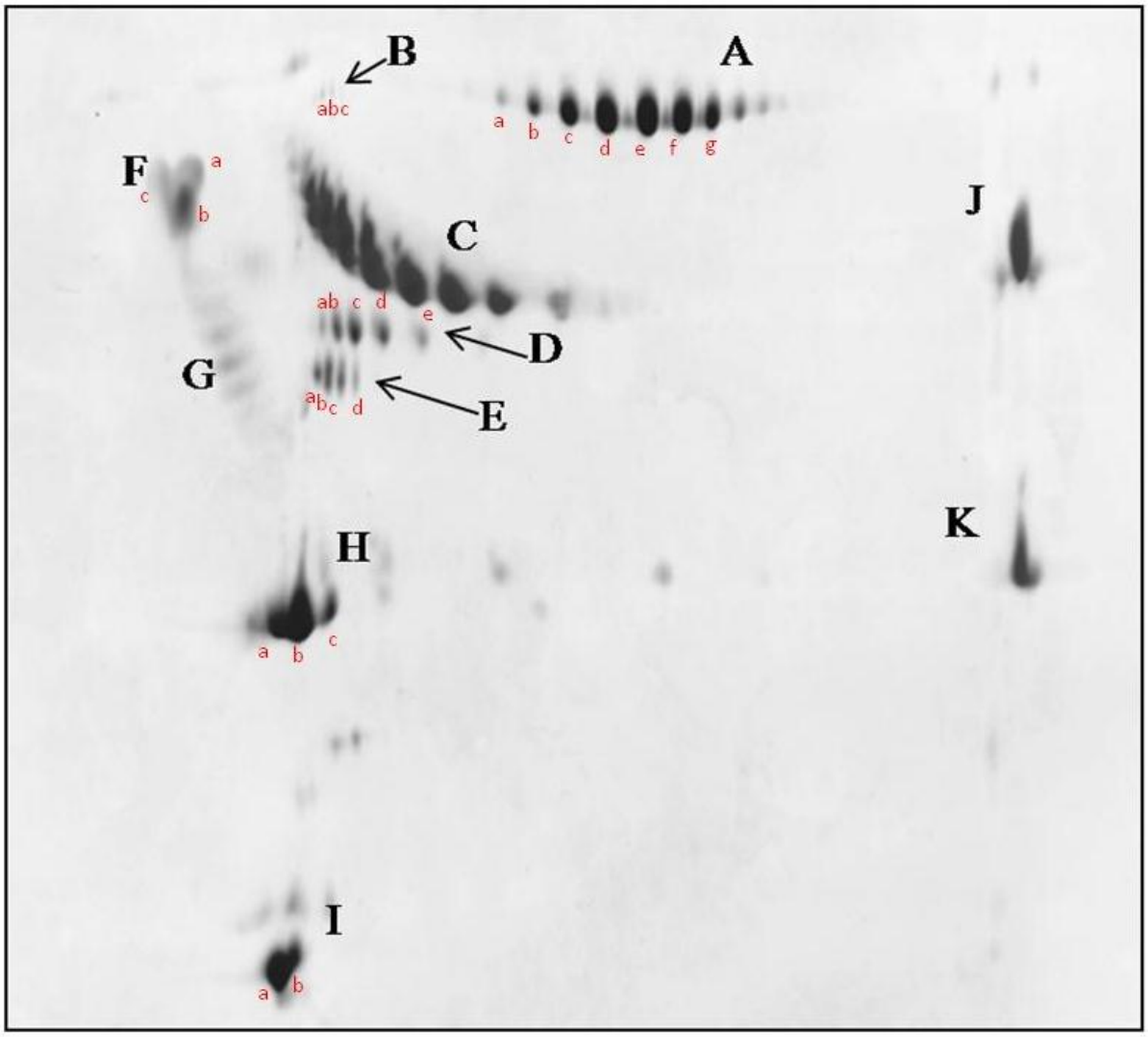


Figure A-1: Labeling key for Appendix Table A-1. For simplicity, the spot map provided is a black and white rendition of the chronic phase oral group 2D SDS-PAGE gels, pH 3-10 non linear, stained with Brilliant Blue R-250.

Table A-1: Complete list of scores for all identified spots.

Map		Protein Name	Acc. No.	Protein score	Pep count	Protein C.I. %	Total Ion score	Total ion C.I.%
ACUTE PHASE								
A	b.	Amy1 protein [Rattus norvegicus]	gi|56971297	354	21	100	145	100
	c.	Amy1 protein [Rattus norvegicus]	gi|56971297	272	19	100	63	99.984
	d.	Amy1 protein [Rattus norvegicus]	gi|56971297	360	21	100	148	100
	e.	Amy1 protein [Rattus norvegicus]	gi|56971297	226	21	100		
	f.	Amy1 protein [Rattus norvegicus]	gi|56971297	285	19	100	103	100
B	a.	chitinase, acidic [Rattus norvegicus]	gi|46485462	105	6	100	66	99.993
	b.	chitinase, acidic [Rattus norvegicus]	gi|46485462	64	5	97.322	28	54.776
	c.	chitinase, acidic [Rattus norvegicus]	gi|46485462	123	9	100	53	99.867
D	a.	deoxyribonuclease I [Rattus norvegicus]	gi|6978769	274	8	100	212	100
	b.	deoxyribonuclease I [Rattus norvegicus]	gi|6978769	289	7	100	219	100
	c.	deoxyribonuclease I [Rattus norvegicus]	gi|6978769	378	10	100	268	100
	d.	deoxyribonuclease I [Rattus norvegicus]	gi|6978769	421	10	100	339	100
	e.	deoxyribonuclease I [Rattus norvegicus]	gi|6978769	199	7	100	102	100
E	a.	cysteine-rich secretory protein 1 [Rattus norvegicus]	gi|12408314	258	11	100	149	100
	b.	cysteine-rich secretory protein 1 [Rattus norvegicus]	gi|12408314	127	6	100	59	99.96
	c.	cysteine-rich secretory protein 1 [Rattus norvegicus]	gi|12408314	86	5	99.984	39	97.209

Table A-1 Continued

E	d.	cysteine-rich secretory protein 1 [Rattus norvegicus]	gi|12408314	240	10	100	147	100
F	b.	Rat submandibular gland secretory protein [Rattus norvegicus]	gi|204477	113	2	100	104	100
H	b.	parotid secretory protein [Rattus norvegicus]	gi|16258825	335	7	100	256	100
CHRONIC PHASE								
A	a.	amylase 1 protein [Rattus norvegicus]	gi|56971297	249	11	100	122	100
	b.	amylase 1 protein [Rattus norvegicus]	gi|56971297	273	14	100	83	100
	c.	amylase 1 protein [Rattus norvegicus]	gi|56971297	809	18	100	565	100
	d.	amylase 1 protein [Rattus norvegicus]	gi|56971297	1150	19	100	896	100
	e.	amylase 1 protein [Rattus norvegicus]	gi|56971297	962	17	100	758	100
	f.	amylase 1 protein [Rattus norvegicus]	gi|56971297	96	7	99.98	50	99.57
	g.	amylase 1 protein [Rattus norvegicus]	gi|56971297	478	15	100	318	100
B	a.	chitinase, acidic [Rattus norvegicus]	gi|46485462	231	9	100	117	100
	b.	chitinase, acidic [Rattus norvegicus]	gi|46485462	150	9	100	61	100
	c.	chitinase, acidic [Rattus norvegicus]	gi|46485462	205	8	100	93	100
D	b	deoxyribonuclease I, isoform [Rattus norvegicus]	gi|149042674	344	7	100	276	100
	c	deoxyribonuclease I, [Rattus norvegicus]	gi|6978769	359	9	100	290	100
	d	deoxyribonuclease I, isoform [Rattus norvegicus]	gi|149042674	259	7	100	167	100

Table A-1 Continued.

F	a	submandibular gland secretory protein[Rattus norvegicus]	gi|204477	78	2	100	63	100
	b	submandibular gland secretory protein[Rattus norvegicus]	gi|204477	160	2	100	153	100
H	a	parotid secretory protein [Rattus norvegicus]	gi|149030955	188	3	100	164	100
	b	parotid secretory protein [Rattus norvegicus]	gi|149030955	130	3	100	107	100
	c	parotid secretory protein [Rattus norvegicus]	gi|149030955	217	3	100	193	100
I	a	cystatin S [Rattus norvegicus]	gi|294577	163	3	100	112	100
	b	cystatin S [Rattus norvegicus]	gi|294577	142	3	100	91	100

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